The Molecular Systematics of *Ulva* Linnaeus and *Enteromorpha* Link (Ulvales, Chlorophyta) from the South Western Cape, South Africa.

By

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Abstract

Both *Ulva* and *Enteromorpha* are very common, ubiquitous, and environmentally important genera of green seaweeds. The evolutionary history of *Ulva* species is poorly understood, stemming from a lack of diagnostic, non-molecular systematic characters. This study addresses the following questions: i) Do the two genera represent distinct entities; ii) What are the relationships among local putative species; iii) Do the recognized species represent monophyletic lineages; iv) How closely are local species related to those from elsewhere? These questions are addressed using nuclear ribosomal DNA ITS sequences from 48 local samples plus data on 16 samples from GenBank. The results add to a growing body of evidence that neither of the two genera is monophyletic, and that the characteristic *Ulva* and *Enteromorpha* morphologies have arisen independently several times throughout the evolutionary diversification of the Ulvaceae. Two major strongly-supported clades, which contained a mix of *Ulva* and *Enteromorpha* morphologies were produced. Perhaps the most interesting finding of this study is the close relationship between *U. capensis* and *U. rigida*, which traditionally are easily distinguished by observing bullet or spindle shaped cells and dentation in the former. Based on ITS data and TCS analysis, the maintenance of the two as separate entities is challenged. South African *U. fasciata* specimens form a well-supported clade separate from *U.rigida/U. capensis*. Specimens originally identified as *E. intestinalis* appear in four lineages in the analysis, including two specimens, which link with *U. rigida/U capensis*. Two others form a clade with an *E. linza* sequence from Genbank, whereas all other local specimens identified as *E. linza* form a separate clade. Although only based on a single sample, *U. uncialis* appears to be a distinct species and not synonymous with *U. rigida* as thought earlier. This study also agrees with earlier studies in supporting the merger between *Blidingia minima* and *B. chadefaudii*, the two were grouped in a well supported (100% bootstrap support) clade.

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Chapter 1: Introduction

Linnaeus in his Species Plantarum (1753) described the genus *Ulva*, together with three other algal genera namely *Fucus*, *Conferva* and *Chara*. The Ulvaceae are morphologically simple ubiquitous macroalgae. *Ulva* and *Enteromorpha* are the most commonly known members, probably because they are prevalent in coastal ecosystems and responsible for "green-tides", an event whereby dense blooms of free-floating algae form as a result of eutrophication.

1.1. Introduction to the Chlorophyta

Hoek *et al.* (1995) provided a recent review of the Chlorophyta, with recent work having been done by Zechmann *et al.* (1990) and based on molecular evidence, combined with morphological and ultrastructural evidence. Earlier hypotheses of the phylogenetic relationships among the green algae have been based largely on ultrastructural studies of the flagellar apparatus, mitosis, and cytokinesis (e.g. Stewart and Mattox, 1978; Mattox and Stewart, 1984; O'Kelly and Floyd, 1984; Hoek *et al.*, 1988, 1992). Stewart and Mattox (1978) erected the class Ulvophyceae, encompassing *Ulva* and related genera that possess a combination of ultrastructural characters distinct from those of the chlorophycean or charophycean lineages.

The class Ulvophyceae (*sensu* Zechmann *et al.*, 1990 and Hoek *et al.*, 1995) was circumscribed to include only two orders, namely the Ulotrichales (or the Codiolales as referred to by Hoek *et al.*, 1995), and the Ulvales. Prior to this, O'Kelly and Floyd (1984) included within the Ulvophyceae orders such as the Siphonocladales (including the Cladophorales), Bryopsidales, Caulerpales, and Dasycladales. O'Kelly and Floyd defined the Ulvophyceae on the basis of ultrastructural characters of the flagellar apparatus (Fig.1.1.), mitosis, and cytokinesis. Within the Ulvophyceae, the reproductive zoids have two or four flagella, with a cruciate, 11 o'clock-5

o'clock configuration of the flagellar apparatus; also a distinct overlap exists between the basal bodies (Fig.1.2.) (O'Kelly and Floyd, 1984; Hoek *et. al.*, 1995).

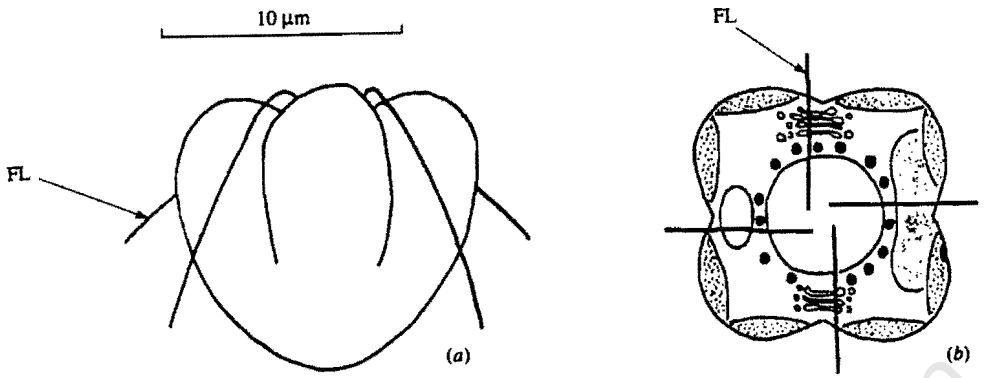

Figure 1.1. A zoid of the cruciate Prasinophyceae type. (a) Gross features of the cell, showing the four flagella and the anterior lobes. (b) Diagram of the cell, as seen from the apex. FL = flagellum (After Hoek *et al.*, 1995).

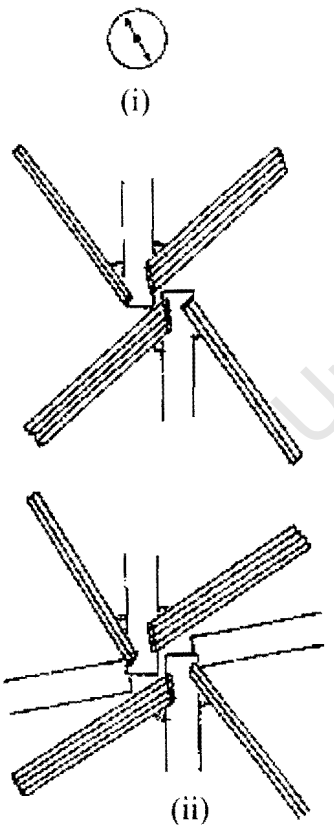

Figure 1.2. The flagellar apparatus of a biflagellate zoid of the cruciate 11 o'clock-5 o'clock type. The basal body and flagellar apparatus is shown from the top (from the anterior of the cell. (i) shows a biflagellate zoid; and (ii) shows a quadriflagellate zoid (After Hoek *et al.*, 1995).

The taxonomic difficulties surrounding *Ulva* and *Enteromorpha* seem to intensify as one goes lower in the classification hierarchy. The difference between the Ulotrichales and the Ulvales is not a clear-cut one, and for a long time, members of the Ulotrichales or the Ulvales have been juggled about. Blackman and Tansley (1902) elevated the Ulvaceae to ordinal rank from Borzi's (1895) Ulotrichales. Blackman and Tansley based their argument on the fact that the Ulvaceae possess a parenchymatous thallus, as opposed to the filamentous state of typical Ulotrichales. Hoek *et al.* (1995) listed some of the characteristics that he considered useful in distinguishing the Ulotrichales from the Ulvales. These features are listed in Table 1.1. below.

Table 1.1. The characteristics that distinguish the Ulotrichales from the Ulvales (*After: Hoek* ***et al.****, 1995)*

ULOTRICHALES	**ULVALES**
Monostromatic	Distromatic
Haplontic life cycle	Diplohaplontic life cycle
Heteromorphic	Isomorphic
Isogamous or Anisogamous	Anisogamous
Zoids with scales	No scales
Possesses a *Codiolum* phase, the only diploid nucleus in the life cycle	

The elevation of the Ulvaceae to ordinal rank was, according to Papenfuss (1960) and Tanner (1981), influenced by the discovery that the life cycle of some species of *Ulva* and *Enteromorpha* was very different from that of the Ulotrichales in that they possess an alternation of isomorphic generations (Fig. 1.3.). A close examination of the sporeling (filamentous) stages of *Ulva* and *Enteromorpha* and the young, uniseriate, branches of *Enteromorpha* reveals a remarkable similarity to filaments of *Ulothrix* Kützing (Papenfuss, 1960, Hoek *et al.* 1995).

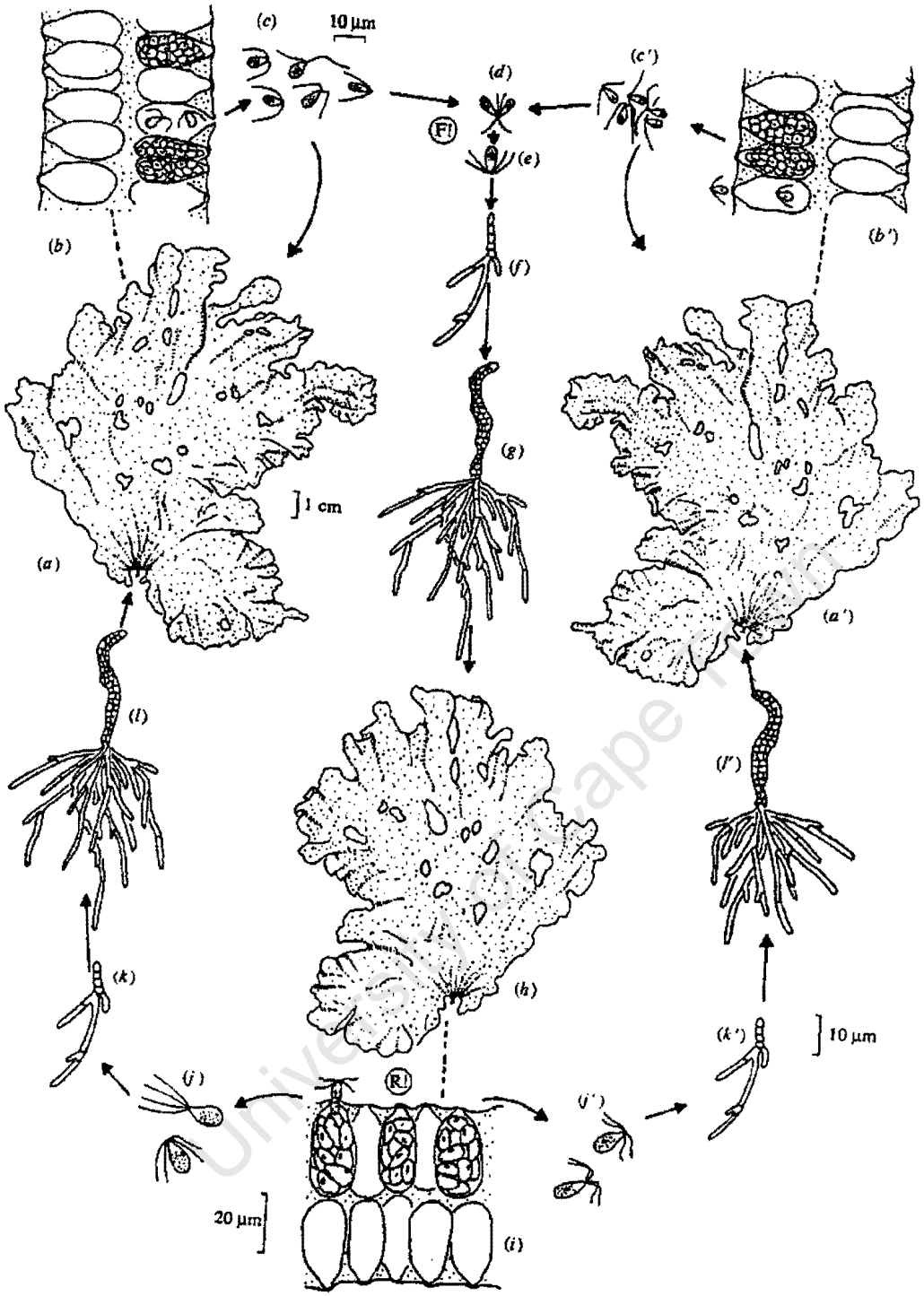

Figure 1.3. The life cycle of *U. lactuca* (Ulvaceae, Ulvales). (*a*, *a'*) Flat blade-like gametophytes. (*b*, *b'*) Division of the cell contents into biflagellate gametes; these are unequal, copulation being anisogamous. (*c*) Female gametes. (*c'*) Male gametes. (*d*) Anisogamous copulation. (*e*) Quadriflagellate planozygote. (*f*) Uniseriate filamentous germling of sporophyte generation attached via branched rhizoids. (*g*) Tubular germling of sporophyte generation. (*h*) Fully developed blade-like sporophyte (diploid). (*i*) Meiotic division of sporophyte cells to form haploid quadriflagellate zoids (meiospores). (*j*, *j'*) Quadriflagellate meiospores. (*k*, *k'*) Uniseriate filamentous germlings of the female and male gametophytes. (*l*, *l'*) Tubular germlings of the female and male gametophytes. F! = fertilization; R! = reduction division (meiosis). Source: Hoek *et al.* (1995). Pp 404-5.

On these grounds Papenfuss endorsed the idea that the Ulvaceae be maintained as a family within the Ulotrichales, but he also acknowledged that they were rather advanced Ulotrichales, a sentiment expressed earlier by Fritsch (1935). More recently however, the placement of the Ulvaceae within the order Ulvales has become widely accepted (Womersley, 1984; Bold and Wynne, 1985; Silva *et al.*, 1996; Stegenga *et al.*, 1997).
The sexually reproducing Ulotrichales are haplontic and possess a *Codiolum* type of zygote, (Hoek *et al.*, 1995). In fact the zygote phase was formerly considered to be a species of that genus (Fritsch, 1935; Burrows, 1991; Hoek *et al.* 1995). *Codiolum* was originally placed by Fritsch (1935) under the Chlorococcaceae (Chlorococcales), a group that includes unicellular and large multinucleate coenobial algae (Fritsch, 1935; Burrows, 1991; Hoek *et al.* 1995). However, it was recognized through culture work that what was initially thought to be the genus *Codiolum*, was a phase in the life histories of other green algae in the Ulotrichales such as, *Spongomorpha aeruginosa*, *Monostroma grevillei* and species of *Urospora* (Burrows, 1991; Hoek *et al.* 1995).

The levels of organization that, according to Hoek *et al.* (1995), occur in the Ulotrichales are: coccoid (*Chlorocystis*), unbranched filamentous (*Ulothrix*), branched filamentous (*Spongomorpha*), unbranched siphonocladous (*Urospora*), branched siphonocladous (*Acrosiphonia*) and thallose (*Monostroma*).

Unlike the Ulotrichales, the sexually reproducing species of the Ulvales have an isomorphic and diplohaplontic life cycle (Fig. 1.3.) (Hoek *et al.*, 1995). But, the diplohaplontic life cycle is thought to have evolved at least four times in green plants, in the classes Ulvophyceae, Cladophorophyceae and Trentepohliophyceae, and in the higher plants (Hoek *et al.*, 1995). This could have serious implications since giving this life history character higher weight could result in these distantly related groups being placed together.

Not all species or varieties in these orders reproduce sexually (Bliding, 1963, 1968; Koeman and Hoek, 1981, 1984), and as a result they are assigned to the particular order because their morphology allies them to the sexual species (Hoek *et al.*, 1995). In addition to *Ulva* and *Enteromorpha*, other genera in the Ulvales are *Blidingia* Kylin, *Percursaria* Bory, *Chloropelta*, *Ulvaria* Ruprecht and *Letterstedtia*. Hoek *et al.* (1995) also included *Acrochaete* within the Ulvales, a view not supported by Stegenga *et al.* (1997). Basing their classification system on that of Womersley (1984), Stegenga *et al.* (1997) placed the genus *Acrochaete* in the order Chaetophorales, which Hoek *et al.* (1995) classified under the class Chlorophyceae rather than the class Ulvophyceae. Stegenga *et al.* (1997) acknowledge using a single class (Chlorophyceae) system.

Many authors recognize the use of a single family (e.g. Bliding, 1963, 1968; Joska, 1992; Stegenga *et al.*, 1997), but some others such as Wynne and Kraft (1981), Silva *et al.* (1996), and Bold and Wynne (1985) distinguish additional families within the Ulvales. Silva *et al*, for instance, in their recent publication, "*Catalogue of the Benthic Marine Algae of the Indian Ocean*", recognized four families within the Ulvales, namely; Capsosiphonaceae, Gomontiaceae, Monostromaceae and Ulvaceae. Some authors however opt for an easy way out and instead completely omit the Family category (Hoek *et al.*, 1995; Lee, 1999). The debate on this topic is beyond the scope of this paper. The genus *Monostroma*, which because of its thalloid morphology was previously included in the Ulvales (Fritsch, 1935), is considered a member of the Ulotrichales on the basis that it has an alternation of heteromorphic phases (Hoek *et al.*, 1995).

Worldwide, the order Ulvales contains about 24 genera and 175 species (Hoek *et al.*, 1995). Almost all are marine, a few

Enteromorpha species being freshwater species (Koeman and Hoek, 1982; Hoek *et al.*, 1995).

1.2 The Ulvaceae

The macroalgae in this family are well known for their simple morphology, which contributes to the difficulties encountered in their identification. Included are all parenchymatous green seaweeds of varied morphology, that are derived from uniseriate filaments by divisions of the cells in two or three planes (Tanner, 1981; Burrows, 1991). A recent phylogenetic study based on nuclear and chloroplast DNA sequences, supported a monophyletic Ulvaceae consisting of *Chloropelta*, *Enteromorpha*, *Percursaria*, *Ulva* and *Ulvaria* (Hayden *et al.*, (2000).

The life cycle of the Ulvaceae (Fig. 1.3.) includes a haploid gametophyte and a diploid sporophyte generation (Hoek *et al.*, 1995). The life cycle is diplohaplontic, with an alternation of isomorphic phases. As outlined by Hoek *et al.* (1995), the haploid gametophyte produces biflagellate gametes that are formed in the marginal cells by mitotic cell division (Fig. 1.3*a*, *a'*). Fusion is anisogamous, and these species are heterothallic. The diploid zygote (Fig. 1.3*e*) germinates with no period of dormancy to give an *Ulothrix*-like germling (Fig 1.3*f*) that develops into a diploid sporophyte (Fig. 1.3*h*) that is morphologically similar to the gametophyte. Through meiosis the cells in the marginal parts of the sporophyte divide to produce a number of quadriflagellate, haploid meiospores (Fig. 1.3*i*). Half of the meiospores grow via *Ulothrix*-like germlings (Fig. 1.3*k'*) into male gametophytes (Fig. 1.3*a'*), while the other half grow into female gametophytes (Fig. 1.3*a*). The blade-like thalli of *Ulva* grow through intercalary cell division, in which the new cell walls are always laid down at right angles to the plane of the thallus.

In *Enteromorpha*, the life cycle is similar, but the uniseriate filaments (Fig. 1.3*f*) become pluriseriate and thus, hollow tubes are produced (Hoek *et al.*, 1995, Pp. 404-5).
Another form of reproduction that is common in the Ulvaceae is parthenogenesis whereby gametes develop into parthenosporophytes (Tanner, 1981; Hoek *et al.*, 1995). Multiplication of the plants by vegetative fragmentation is common in this group (Bonneau, 1978; Tanner, 1981; Hoek *et al.*, 1995). Sterile strains of *Ulva* have also been isolated in the non spore-producing *U. pertusa* (Migita, 1985).

1.2.1 Habitat and distribution

The Ulvaceae are characteristically inhabitants of the intertidal (but also the subtidal). They are normally epilithic but some species, such as *U. rhacodes*, may be epiphytic (Stegenga *et al.* (1997). They often form extensive mats in highly dynamic environments, for instance where the rocks or boulders are frequently covered and then uncovered by shifting sands in the intertidal zone (Dickinson, 1963).

They command a high growth rate and the ability to take up nutrients rapidly. As a result, *Ulva* has proliferated in many areas that have had a nutrient input, especially from anthropogenic activities. A feature of nuisance growths of *Ulva* in enclosed waters such as harbours is that *Ulva* makes up most of the drift plants, which may smother other benthic communities or be cast ashore where they decompose. Due mainly to the production of hydrogen sulphide, they become a nuisance (Dickinson, 1963; Lee, 1999). Many species in the Ulvaceae can continue to live in floating communities when detached from the surface on which they grow (Dickinson, 1963).

The distribution of these genera is world wide, occurring from polar seas almost to the limit of seaweed vegetation, and in the tropics (Dickinson, 1963). A few species of *Enteromorpha*

do just as well in fully saline as in freshwater conditions (Dickinson, 1963; Koeman and Hoek, 1982).

Due to the difficulties in the identification of members of this group, many species names have been misapplied (Silva *et al.*, 1996) and this has resulted in artificially inflated ranges for many of the species. For instance *U. lactuca* Linnaeus is a name, which has often been misapplied. Stephenson (1948), Stegenga *et al.* (1997) and Silva *et al.* (1996) listed many such cases.

1.2.2. Social and economic impacts of the Ulvaceae

Ulva species are increasingly suggested for use as biofilters for marine fishpond effluents. The alga is very efficient in removing nitrogenous compounds from wastewater, and the effluents in turn support the growth of *Ulva* (Cohen and Neori, 1991; Neori *et al.*, 1991, 1996; Jimenez Del Rio *et al.*, 1996; Goldberg *et al.*, 1998). Indeed Goldberg *et al.* (1998) recorded a removal of 95% of the ammonia contained in the effluent in which *Ulva rigida* was grown. The resulting protein rich seaweed biomass may in turn be used as food for aquaculture fisheries such as abalone, *Haliotis midae* (Simpson and Cook, 1998; Steyn, 2000) and the fish, *Sparus aurata* L. (Neori *et al.* 1996).

Malea and Haritonidis (2000) found significant positive correlations between lead (Pb) concentrations in the alga with concentrations in seawater. They concluded that *U. rigida* C. Agardh could be used as an indicator species to assess metal pollution in marine environments, especially for Pb.

In China, *Ulva lactuca* has been collected by fishermen along the coasts of China and sold in markets both as food and as medicine. *Enteromorpha* is also sold in the markets of China and Japan as a common article of food (Dickinson, 1963). *Ulva* species have also been collected for food in Latin America,

particularly in Chile, and Lee (1999) reported that this alga is consumed in Scotland.

1.2.3 Morphological plasticity

Morphologically, *Ulva* has been identified by its fully distromatic thallus, distinguishing it from its closest relative, *Enteromorpha* (the gut weed), which is tubular. However, the distinction between these two genera has been challenged by several authors such as Bonneau (1977), who demonstrated that *Ulva* and *Enteromorpha* may be isomorphic in a given environment. Bonneau found that a single swarmer population could produce all *Enteromorpha*-like plants, all *Ulva*-like plants, or a mixture of types. In some instances both growth forms were also found growing on the same blade.

Ulva is notorious for its taxonomic difficulties (Papenfuss, 1960; Bliding, 1963, 1968; Steffensen, 1976; Tanner, 1986; Koeman and Hoek, 1981; Hoeksema and Hoek, 1983; Joska, 1992). This, as Papenfuss (1960) noted, is surprising considering that *Ulva* is such a ubiquitous alga and was one of the first four algal genera to be described in Linnaeus' *Species Plantarum*. Most of the taxonomic problems have been associated with the morphological plasticity that is widespread in the genus (Kapraun, 1970; Steffensen, 1976; Bonneau, 1977). Koeman and Hoek (1980) agreed with Bliding (1963, 1968) in finding it very unrealistic to base the taxonomy of *Ulva*, *Enteromorpha* and related genera on herbarium material. This is because many taxonomic characters such as cytology, reproduction and the natural variability cannot be adequately studied on the basis of preserved material. After a series of studies by other researchers (e.g. Bonneau, 1977; Kapraun, 1970; Steffensen, 1976; Mshigeni and Kajumulo, 1979) it was found that even with fresh material the identification of *Ulva* species remains difficult due to morphological plasticity. Morphological and cytological characteristics that are used today have been shown to vary with season, wave energy, latitude and

geographical location, even within a single population at a given time (Steffensen, 1976; Tanner, 1986; Phillips, 1984, 1988; Woolcott & King, 1999).

Burrows (1991) found that the growth form, and the branching pattern in particular, of *Enteromorpha* are not foolproof as identification tools. She arrived at this conclusion after observing that temperature has an apparent effect on the branching pattern of *Enteromorpha*. Reed and Russell (1978) also reached the same conclusion using *E. intestinalis*; they found that changes in salinity could initiate proliferation. A study conducted by Tanner (1986) found wave energy to be a factor affecting the size and morphology of *U. californica* plants. Wave energy was also found to be responsible for stunted growth in *U. fasciata* Delile (Mshigeni and Kajumulo, 1979) and *U. fenestrata* (Titlyanov *et al.*, 1975).

Morphological differences between the species are small and difficult to detect (Bliding, 1963). Another problem is that the morphological characters are quantitative, each showing a graded series with overlap between species. This therefore leaves a lot of room for subjectivity among taxonomists with regard to the recognized species (Bliding, 1963, 1968).

1.3. Molecular phylogenetic studies

The confusion in the taxonomy of these two genera has also been revealed by molecular analyses. These showed that the two genera are polyphyletic and that species from one genus are often nested among those of the other (Tan *et al* 1998; Blomster, 1999*a*; Woolcott and King 1999). Some species were also shown to be polymorphic (Malta *et al.*, 1999; Woolcott and King 1999).

No molecular analyses have ever been carried out on South African species. Analyses of DNA sequences have been shown to be very useful for examination of relationships at the level

of genus and below in other members of the Ulvophyceae. Most such approaches utilized the sequences of the 'internal transcribed spacer' (ITS1 and ITS2). These noncoding gene regions are located between the nuclear 18S and 5.8S, and 5.8S and 26S ribosomal genes, respectively. In other words the complete ITS region includes the more conserved 5.8S ribosomal RNA gene (see Fig. 2.3.1.). Some examples of phylogenetic studies where molecular approaches have been successful are: within the Cladophorales (Marks and Cummings, 1996; Bakker *et al.* 1992, 1995; Kooistra *et al.*, 1992, 1993), the Caulerpales (Pillman *et al.*, 1997), and the Ulvales, (Leskinen and Pamilo, 1997; Blomster *et al.*, 1998, 1999; Coat *et al.*, 1998; Malta *et al.*, 1999; Woolcott and King, 1998, 1999; Woolcott *et al.*, 2000).

1.3.1. Molecular markers

The ribosomal ITS sequence is a fast-evolving DNA region, and that is why it is such a useful tool for the study of interspecific variation as discussed above (Leskinen and Pamilo, 1997, Judd *et al.*, 1999). The reason why the ITS has been so successful in phylogeny reconstruction is that the spacer undergoes rapid concerted evolution whereby the highly repetitive sequences are homogenized (Judd *et al.*, 1999; Harris and Crandall, 2000). During this process any copy of the sequence that undergoes mutation can be corrected to match the remaining copies. However, the reverse is also possible (Judd *et al.*, 1999; Harris and Crandall, 2000), so that point mutations are rapidly fixed. It is very important to note at this stage that ITS sequences do not show homogenization in some lineages, and this can produce spurious phylogenetic inferences (Buckler *et al.*, 1997). Other genes such as the 5.8S or the chloroplast gene *rbcL*, have a slower rate of change, and as a result they are more useful for deeper phylogenetic studies (Leskinen and Pamilo, 1997; Judd *et al.* 1999).

1.4. Objectives of this study

South African species of *Ulva* and *Enteromorpha* have never been investigated at a molecular level, and it is hoped that this study will shed some light on the taxonomic status of the local species of this green algal group. The objectives of this study are as follows:

1. To test the monophyly of the recognized genera *Ulva* and *Enteromorpha* on the basis of ITS (Internal Transcribed Spacers) sequences
2. To compare the results of molecular analyses of local species to those obtained elsewhere
3. To investigate the existing species level classification of these taxa.

Chapter 2: Materials and methods

2.1. Collection

The plant specimens used in this investigation were intertidal (no estuarine samples) and were mostly collected from various populations along the west and south coasts of South Africa. In addition, two specimens were collected from the Namibian coastline (Fig. 2.1.1). The collection details are given in Table 2.1.1. Most of the populations were found attached to rock in the intertidal zone, while others were free living. The free-living populations were found in sheltered bays such as Simonstown and Gansbaai harbours. As reported in earlier studies (Dion & Le Bozec, 1996), the free-living forms are a major constituent of green tides that occur in many parts of the world, and they are a symptom of eutrophication. Most of the sampled sites were in close proximity to the laboratory, and as a result they were transported in plastic bags containing seawater. The samples from localities further afield were pickled in 10% formalin for a few hours before being dried on herbarium sheets.

The materials destined for DNA extraction were dried in silica gel (often in the field), and these were clearly labelled to make sure that the material is from the same sample used to identify it. These samples were cleaned of any obvious epiphytes or alternatively only the clean blades were used. However, samples of the smaller specimens, e.g. U. *uncialis* (Kützing) Montagne or of tubular *Enteromorpha* species, were not always easily cleaned.

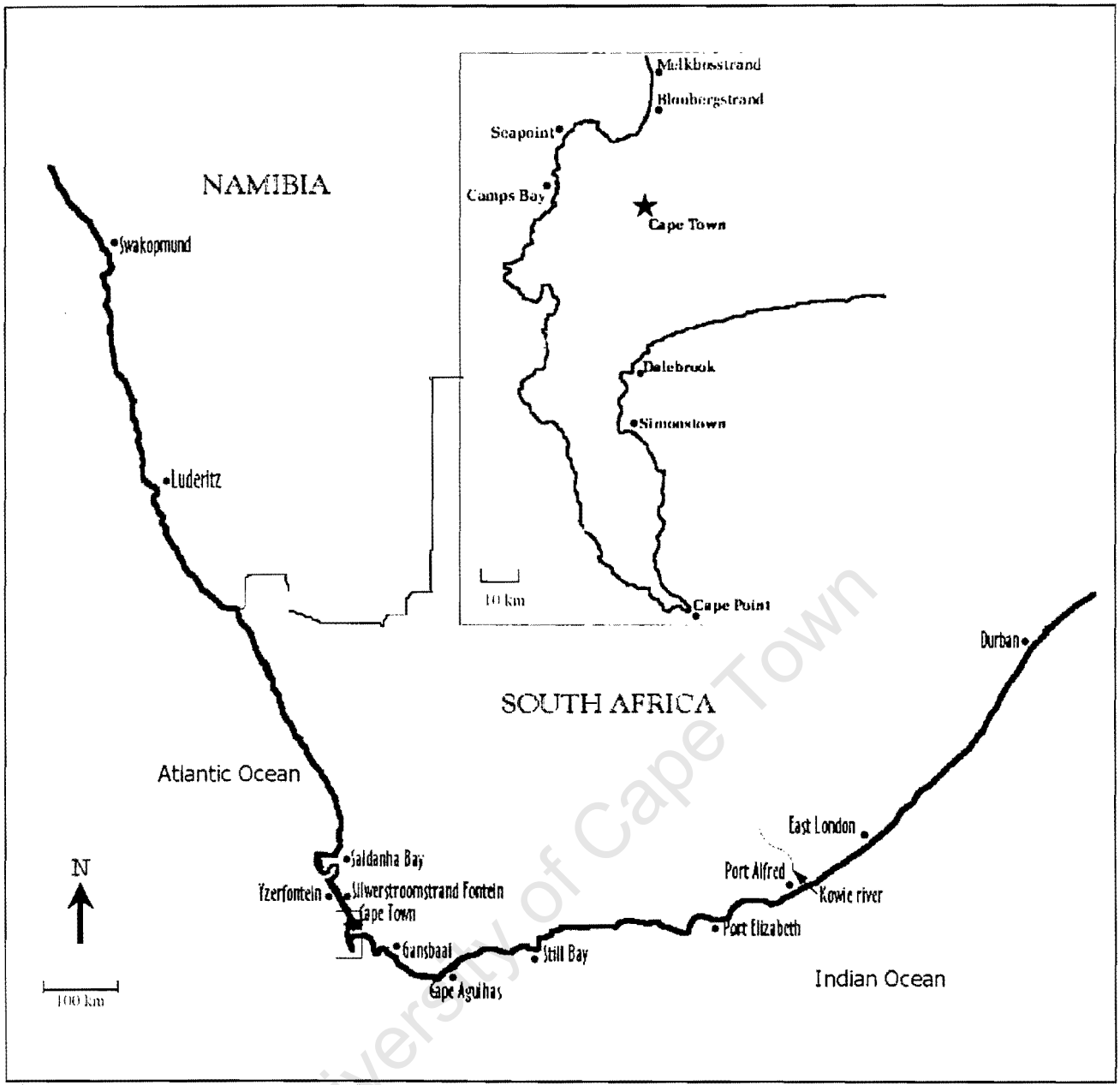

Figure. 2.1.1. The Map of South African (and Namibian) west coast showing some of the sites mentioned in the text.

An additional batch of sequences was obtained from GenBank and these are listed in Table 2.1.2. These were important to allow for a comparison of this study to published work. All but one of the GenBank sequences that could be aligned were from a single publication, Tan *et al.*, (1999). The odd one was an unpublished one, but deposited by researchers from the same institution (Tan and Sluiman, unpubl.).

Table 2.1.1. Samples of *Ulva*, *Enteromorpha* and *Blidingia* sequenced, along with collection locality, grid references, Date of Collection, herbarium accession number.

Area of collection	Collector	Grid references	Species name	Analysis code	Sample no/Herb spec. no	Date of col.
Gansbaai harbour	L. Kandjengo	3419CB	*U. capensis*	U03	LK 6	31/7/2001
Riet river	J.J. Bolton	3327CA	*U. fasciata*	U07	LK 66	7/7/2001
Gansbaai I&J farm	L. Kandjengo	3419CB	*U. lactuca*	U09	LK 2	31/7/2001
Groot Jongensfontein	J.J. Bolton & R.J. Anderson		*U. rigida*	U10	No Herb. specimen	10/4/2001
Saldanha Bay harbour	R.J. Anderson	3217DD	*E. linza*	U11	LK 29	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*U. capensis*	U13	LK 14	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*E. linza*	U14	LK 13	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*U. rigida*	U15	LK 17	7/8/2001
Gansbaai I&J farm	L. Kandjengo	3419CB	*U. capensis*	U16	LK 4	31/7/2001
Gansbaai I&J farm	L. Kandjengo	3419CB	*E. intestinalis*	U17	LK 11A	31/7/2001
Gansbaai I&J farm	L. Kandjengo	3419CB	*E. linza*	U18	LK 10	31/7/2001
Swakopmund, Namibia	L. Kandjengo	2014DA	*U. rigida*	U19	LK 9	4/7/2001
Gansbaai I&J farm	L. Kandjengo	3419CB	*Ulva sp*	U21	LK 5	31/7/2001
Silwerstroomstrand fontein	L. Kandjengo	3318CB	*E. linza*	U23	LK 21	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*U. capensis*	U24	No Herb. Specimen	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*E. linza*	U26	LK 15	7/8/2001
Silwerstroomstrand fontein	L. Kandjengo	3318CB	*U. capensis*	U27	LK 22	7/8/2001
Melkbosstrand	L. Kandjengo	3318CB	*E. intestinalis*	U28	LK 23	7/8/2001
Melkbosstrand	L. Kandjengo	3318CB	*E. linza*	U29	LK 24	7/8/2001
Melkbosstrand	L. Kandjengo	3318CB	*U. capensis*	U30	LK 25	7/8/2001
Silwerstroomstrand fontein	L. Kandjengo	3318CB	*U. capensis*	U31	LK 20	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*U. rigida*	U32	LK 16	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*U. capensis*	U34	LK 11B	7/8/2001
Yzerfontein	L. Kandjengo	3318AC	*E. linza*	U35	LK 19	7/8/2001
Still bay (Shelly beach)	L. Kandjengo	3421AD	*U. uncialis*	U36	LK 64	9/4/2001
Bloubergstrand	L. Kandjengo	3318CD	*U. capensis*	U37	LK 26	8/8/2001
Bloubergstrand	L. Kandjengo	3318CD	*U. rigida*	U38	LK 27	8/8/2001
Bloubergstrand	L. Kandjengo	3318CD	*E. linza*	U39	LK 28	8/8/2001
Jacobsbaai	R.J. Anderson	3217DD	*E. intestinalis*	U40	LK 31	8/8/2001
Seapoint north	L. Kandjengo	3318CD	*E. linza*	U41	LK 32	13/8/2001

Table 2.1.1. (*Continued*)

Seapoint north	L. Kandjengo	3318CD	*E. intestinalis*	U43	LK 34	13/8/2001
Seapoint north	L. Kandjengo	3318CD	*E. flexuosa*	U45	LK 36	13/8/2001
Seapoint north	L. Kandjengo	3318CD	*U. capensis*	U46	LK 37	13/8/2001
Seapoint north	L. Kandjengo	3318CD	*U. capensis*	U47	LK 38	13/8/2001
Seapoint south	L. Kandjengo	3318CD	*U. capensis*	U49	LK 40	13/8/2001
Seapoint south	L. Kandjengo	3318CD	*E. linza*	U50	LK 41	13/8/2001
Seapoint south	L. Kandjengo	3318CD	*E. intestinalis*	U51	LK 42	13/8/2001
Seapoint south	L. Kandjengo	3318CD	*E. intestinalis*	U52	LK 42	13/8/2001
Seapoint south	L. Kandjengo	3318CD	*E. compressa*	U53	LK 43	13/8/2001
Camps Bay	L. Kandjengo	3318CD	*E. linza*	U54	LK 44	13/8/2001
Camps Bay	L. Kandjengo	3318CD	*U. capensis*	U55	LK 45	13/8/2001
Dalebrook	L. Kandjengo	3418AB	*E. linza*	U59	LK 49	17/9/2001
Dalebrook	L. Kandjengo	3418AB	*E. linza*	U60	LK 50	17/9/2001
Dalebrook	L. Kandjengo	3418AB	*E. compressa*	U61	LK 51	17/9/2001
Simonstown harbour	L. Kandjengo	3418AB	*U. lactuca*	U63	LK 53	17/9/2001
Dalebrook	L. Kandjengo	3418AB	*U. rhacodes*	U64	LK 48	17/9/2001
Jongensfontein (Still bay)	L. Kandjengo	3421AD	*U. fasciata*	U65	LK 55	16/10/2001
Jongensfontein (Still bay)	L. Kandjengo	3421AD	*U. fasciata*	U66	LK 56	16/10/2001
Jongensfontein (Still bay)	L. Kandjengo	3421AD	*B. minima*	U67	LK 63	16/10/2001

Table 2.1.2. Sequences of *Ulva*, *Enteromorpha*, *Blidingia* and *Monostroma* obtained from GenBank with collection locality, author and EMBL accession number.

Sample	Collection locality	EMBL Accession No.	Author	Note
U. rigida	Skara Brae, Orkney, Scotland	AJ234319	Tan *et al.* (1999)	
U. lactuca	Redpoint, Wester Ross, Scotland	AJ000208	Tan *et al.* (1999)	This GenBank accession no. (AJ000208) was assigned to *U. rigida* in the cited literature.
U. fenestrata	North Boardman St. Park,	AJ234316	Tan *et al.* (1999)	
U. scandinavica	Langstone Harbour, Portsmouth, England	AJ234318	Tan *et al.* (1999)	
U. taeniata	Seal Rock, Oregon, U.S.A	AJ234320	Tan *et al.* (1999)	
E. linza	Ythan Estuary, Aberdeenshire, Scotland	AJ000203	Tan *et al.* (1999)	
U. californica	Otter Crest, Oregon U.S.A	AJ234315	Tan *et al.* (1999)	
E. intestinalis	Gills Bay, Highland, Scotland	AJ234299	Tan *et al.* (1999)	
E. intestinalis	Bamfield, British Columbia	AJ234300	Tan *et al.* (1999)	
U. pseudocurvata	Ythan Estuary, Aberdeenshire, Scotland	AJ234312	Tan *et al.* (1999)	
U. pseudocurvata	Ythan Estuary, Aberdeenshire, Scotland	AJ234313	Tan *et al.* (1999)	
U. pseudocurvata	Ythan Estuary, Aberdeenshire, Scotland	AJ234314	Tan *et al.* (1999)	
E. compressa	Quarterland Bay, Strangford Lough, Northern Ireland	AJ234301	Tan *et al.* (1999)	
E. compressa	Portaferry, Strangford Lough, Northern Ireland	AJ234302	Tan *et al.* (1999)	
B. chadefaudii	Carnalea, Belfast Lough, Northern Ireland	AJ012309	Tan *et al.* (1999)	
M. grevillei		AJ000205	Tan and Sluiman	Unpublished

Note: Many specimens (sequences) were downloaded from the Genbank but they could not be aligned and were thus left out from the analyses.

2.2. Morphology

Morphological observations made on the algae during the collection included: habit, thallus texture, colour, dentation (where microscopic), branching patterns. Working on European species of *Ulva*, Bliding (1968) proposed a number of identification criteria by which he recognised eight species in Europe. The characters used by Bliding were mainly microscopic: cell size and shape (in cross section and surface view of at least five randomly chosen cells), thallus (lamina) thickness, cell arrangement in surface view, and number of pyrenoids. For each plant in the current study several parts of the thallus were examined. These were the upper (marginal), mid and basal (just above the holdfast or the rhizoidal section) regions (Fig. 2.2.1.), except for the free-floating samples which had no holdfast and, hence, no basal section. Thickness and cell dimensions were measured with an ocular micrometer at 400× power. The study included 71 specimens and these were measured for the above-mentioned characters and then compared to the existing records. Permanent slides were prepared using 50% corn syrup (50% Karo® corn syrup, 1% Aniline blue, 3% 1N HCl, 46% H_2O) and Fast green. Voucher specimens of the studied samples were deposited in the Bolus Herbarium (BOL), University of Cape Town, South Africa.

Identifications of local material were made using Joska (1992) and Stegenga *et al.* (1997). Where necessary, additional references such as Bliding (1963, 1968), Koeman and Hoek (1981, 1984) Hoeksema and Hoek (1983), Womersley (1984), Phillips (1988), Burrows (1991), and Adams (1994) were consulted.

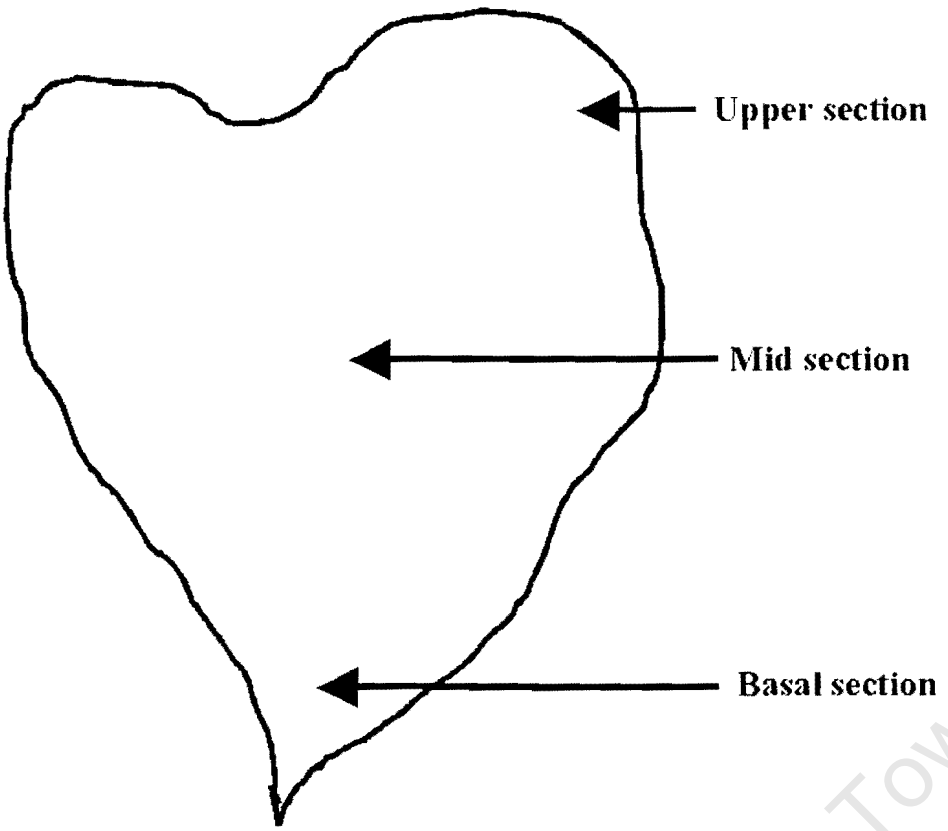

Figure. 2.2.1. The schematic drawing of *Ulva* showing the regions of the thallus as described in the text.

The taxonomy of *Ulva* species from the South Western Cape has recently been studied by Joska (1992), and descriptions of all species occuring on the west coast have been documented by Stegenga *et al.* (1997). The following six *Ulva* species have been identified thus far; *U. capensis* Areschoug, *U. fasciata*, *U. lactuca*, *U. rigida*, and *U. rhacodes* (Holmes) Papenfuss. No formal description exists for the form that Stegenga *et al.* (1997) referred to as "*U. uncialis*", meaning an inch long/high. Believing that the form was a dwarf form of *U. rigida*, Stegenga *et al.* placed this form accordingly. Six *Enteromorpha* species have been recorded locally: *E. intestinalis*, *E. linza* [Linnaeus] J. Agardh, *E. atroviridis*, *E. compressa*, *E. flexuosa* and *E. prolifera*. The species collected are listed in Appendix I with their synonyms.

2.3. Molecular data

Traditionally taxonomists have relied on morphological, anatomical and cytological data to distinguish species. However, since the introduction of the 'polymerase chain reaction' (PCR) and DNA sequencing the use of phylogenetic studies has increased and indeed this field is moving at a very fast pace, with new molecular markers being characterised continuously. Among problems associated with DNA sequencing, perhaps the most crucial one is the choice of a suitable molecular marker. For our investigation, the ITS gene region (Fig. 2.3.1) was chosen as discussed in Chapter 1. This also makes it possible for us to compare our results to those from earlier studies.

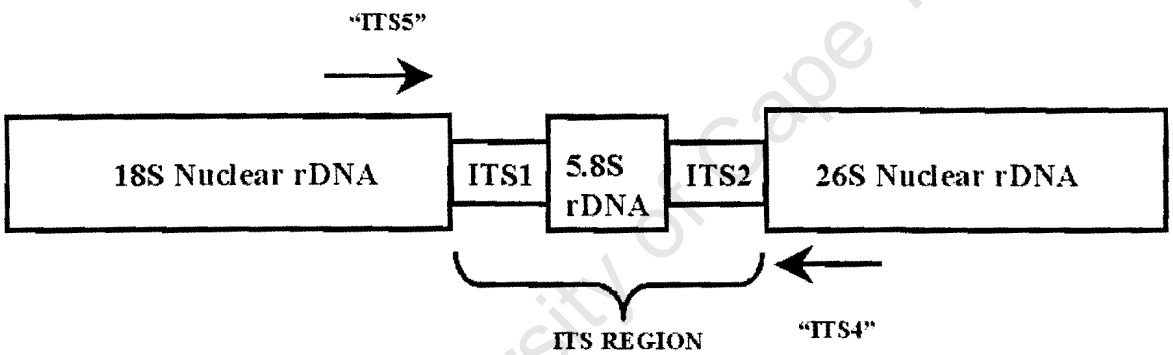

Figure 2.3.1. Schematic map of the *Ulva* rDNA region showing the DNA genes separated by the internal transcribed spacers (ITS). The different primers used are shown by arrows.

2.3.1 Total DNA extraction and purification

The DNA extraction protocol used in this investigation is a modification of Gawel and Jarret (1991). Small quantities of dried material (up to approx. 20mg) were placed into well-labelled 1.5ml microcentrifuge tubes. The tubes were then immersed into liquid nitrogen after which the samples were finely ground using a plastic pestle. Meanwhile, 700µl of 2 X CTAB extraction buffer was mixed with 1µl of -mercaptoethanol for each sample. The mixture was then placed in a Nunc-type tube, and put into a pre-heated bath. 700µl of the pre-heated

CTAB buffer was added to the frozen ground material. The resulting mixture was thoroughly mixed before being incubated in a water bath at 65 C for 30 minutes. The material was then removed from the water bath and 600µl of chloroform: isoamyl alcohol was added to each tube and mixed by inversion for 5 minutes, and centrifuged at 12,000rpm for five minutes. After centrifuging, the supernatant was pipetted (taking note of the volume) into a clean 1.5ml microfuge tube. An equal volume of ice-cold isopropanol was added to the supernatant and briefly mixed by inversion. The samples were stored in the fridge for at least one hour to precipitate the DNA. The chilled samples were spun at 12,000rpm for five minutes to recover the DNA pellet. The isopropanol was discarded by tipping it carefully out of the tube. The open tubes were inverted onto a tissue paper to allow residual liquid to drain out. Ten minutes or so, the tubes were mopped for any droplets. Pellets were washed in 250µl of 75% ethanol and spun at 12,000rpm for 2-3 minutes. The ethanol was discarded and the tubes inverted onto the paper towel to allow the ethanol to drain. Samples were dried in vacuum, after which the DNA was re-suspended in 50µl of sterile, distilled water. Total DNA was then run on a 1% agarose gel to assess the quality of the extract.

2.3.2. DNA amplification and sequencing

The ITS region was amplified by polymerase chain reaction (PCR). Primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al.*, 1990) were used to amplify the ITS region. For PCR amplification a Hybaid PCR Sprint thermal cycler was used. The cycle was two minutes of initial denaturation at 97°C, followed by 30 cycles of one minute at 97°C, followed by one minute annealing at 52°C, and two minutes extension at 72°C and a final extension period of seven minutes at 72°C. PCR reactions were run in 50µl volumes, with each of the following components: 1.5 µl of each 10µM primer, 5.0µl of 50mM $MgCl_2$, 5.0µl of 10 X NH_4 buffer, 2.0µl of

2.5mM DNTP's, 0.3µl of 1.5 units Taq polymerase, 29.7µl nP H_2O; 5.0µl DNA template. A negative control including all components except for DNA was also included in each PCR set. The inclusion of a negative is crucial for detection of any contamination.

In order to determine the size of the DNA fragment, and to assess the quality of the PCR products, they were run along side a lambda-ladder on a 1% agarose gel as discussed above. PCR products (approx. 600 bp in length) were cleaned using the QIAquick™ (Qiagen) PCR purification kit according to the manufacturers instruction, the products were eluted with deionised water or the manufacturers elution buffer. Some of the PCR products produced double bands on the agarose gel and these samples were discarded as a result.

The purified double stranded PCR products were sequenced using the ABI PRISM™ Big Dye Terminator Cycle Sequencing kit, as per the manufacturer's instructions using the amplification primers as sequencing primers. The cycle sequencing reactions were carried out in 25 cycles of denaturation at 96 C for 30 seconds, 50 C for 15 seconds and 60 C for 4 minutes. Cycle sequencing products were resolved on an ABI 3900 Automated Sequencer. The sequences were assembled and checked for inaccurate base calling using SegMan II (Laser Gene System software, DNAStar, Inc), and assembled sequences were aligned manually using MegAlign (Laser Gene System software, DNAStar, Inc). Maclade was used to edit the alignment, and exclude ambiguously aligned regions.

2.3.3. Phylogenetic analyses

Two representatives from the same family as *Ulva* and *Enteromorpha* (*Blidingia chadefaudii* [Tan *et al.*, 1999] and *B. minima*; Ulvaceae, Ulvales), as well as a parenchymatous green alga (*Monostroma grevillei* [Tan *et al.*, 1999]; Monostromaceae,

Ulotrichales), served as outgroup taxa in all phylogenetic reconstructions, based on the life history, ontogeny, and ultrastructure (Bliding, 1968; Hoek *et al.*, 1995). *Monostroma grevillei* was used to root the trees.

a) Unweighted parsimony analyses

All phylogenetic analyses were conducted in PAUP 4.0b4 (Swofford, 1999). Parsimony analyses were conducted with the heuristic search function. Strict consensus trees were used to summarise the most parsimonious trees (MPT). 40 bases at the 3′ end and a further 28 bases of the amplified segment were excluded because these were missing in most of the species sampled.

The whole set of sequences was initially analysed by the parsimony optimality criterion. The initial heuristic criterion was conducted using 10,000 replicates of random taxon addition. At each replicate, a maximum of two trees was saved. The saved trees were used as starting trees in a second round of tree searching. Each was swapped to completion with TBR branch swapping, and branches were collapsed if maxlength=0. All most parsimonious trees were saved, up to a maximum of 25,000.

The number of characters in the alignment was 604 and these were of the unordered type. All were accorded equal weights. Three hundred and ninety five characters were constant and 70 variable characters were parsimony-uninformative; thus only 139 characters were parsimony-informative. In all the analyses gaps were treated as missing.

The *Ulva capensis/rigida* grouping was analysed separately in order to get a clearer picture of the relationship among these two putative species. Some regions excluded in the overall analysis because of alignment ambiguity, could easily be

aligned within smaller groups. Inclusion of these regions could potentially lead to greater phylogenetic resolution within such groups. In this analysis, among the samples that were identical, some were excluded inorder to speed-up the analysis. A heuristic search was conducted using 10,000 replicates of random taxon addition. All most parsimonious trees produced by TBR branch swapping were saved. The branches were collapsed if maxlength=0. The total number of characters was 596. These were of the unordered type and were accorded equal weights. Three hundred and ninety seven of these characters were constant, 142 of the variable characters were uninformative and only 57 (which is 82 less then those obtained in the main analysis) were parsimony informative.

A separate analysis was also conducted to assess the relationships within *Enteromorpha*. As in the case of the *U. rigida/capensis* analysis this would reduce alignment ambiguity. A heuristic search was conducted using 10,000 replicates of random taxon addition. All most parsimonious trees produced by TBR branch swapping were saved. The branches were collapsed if maxlength=0. The number of characters in the alignment was 620 and these were of the unordered type. All the characters were accorded equal weights. Three hundred and seventy six characters were constant and 137 variable characters were parsimony-uninformative; thus only 107 (32 less than the number of informative characters obtained in the main analysis) characters were parsimony-informative.

The bootstrap method of Felsenstein (1985) with 100 replicates was used to estimate the reliability of the groupings in the parsimony analyses.

b) TCS analysis

Samples of closely related species, for example those that produced polytomies under maximum parsimony, were reanalysed

using TCS, a computer program that was developed by Templeton *et al.* (1992) to estimate gene genealogies. The TCS software program is more suitable for population level analyses (Clement *et al.* 2000). Under normal parsimony approaches, ancestors are presumed extinct with population level phenomena, however, this is usually not the case, and "ancestral" and "descendant" haplotypes may occur in the same population. The program identifies haplotypes where differences do not exceed some 95% probability of parsimony criterion. The resulting graph also clearly indicates the number of steps connecting two haplotypes (Clement *et al.* 2000).

Chapter 3: RESULTS

3.1 Morphology

The difficulty of identifying *Ulva* and *Enteromorpha* species was felt in this study. The first step in every identification session was to place the collection into groups according to their thallus morphology. This was then followed by the anatomical and cytological studies which when combined could delineate the known species. All the characters used are listed in Table 3.1.1. below. The first grouping was that made up of the distromatic flat sheet-like fronds (*Ulva*) and the other being the monostromatic tubular forms (*Enteromorpha*). The *Ulva*-like forms were then separated into linear (*U. fasciata* [Fig. 3.1.1], *U. nematoidea* Bory [Fig. 3.1.2], *U. lactuca* [Fig. 3.1.3]) and the non-linear type (*U. rigida* [Fig. 3.1.4], *U. capensis* [Fig. 3.1.5], *U. uncialis* [Fig. 3.1.6], *U. rhacodes*, and a single specimen that could not be identified to the species level [Fig. 3.1.7]). This specimen was collected from the I&J abalone farm Gansbaai where it is grown in tumble culture. It closely resembled *Ulva* lactuca but differed by being consistently wrinkled at perforations, and with the number of pyrenoids ranged between one and four, whereas in *U. lactuca* the number rarely exceeded two. The specimen was also much thicker (above 53 μm) than *U. lactuca* (38-86 μm). In comparison with other *Ulva* species, this specimen exhibited cells that, in transverse section, were more rounded and the thallus had no dentation. *Enteromorpha linza* [Fig. 3.1.8] looks a lot like *Ulva* species, the only difference is that it is much softer to the feel and has a hollow stipe.

The *Enteromorpha*-like forms were separated into two groups, the branched (*Enteromorpha compressa* [Fig. 3.1.9], *E. flexuosa*) and the unbranched forms (*E. intestinalis* [Fig. 3.1.10]). *E. intestinalis* may, however, be branched

occasionally. The unbranched forms also included a form that corresponded to *Blidingia minima* (Nägeli ex Kützing) Kylin [Fig. 3.1.11].

The cell shape character was used to separate *U. capensis* from other non-linear forms by having spindle or bullet shaped cells in transverse section, especially in the basal region. *U. rigida* and *U. capensis* both had much bigger cells (up to 53 and 67 μm high, respectively). The thallus of the two species was also generally much thicker (up to 209 and 247 μm, respectively.). The thallus thickness of *U. lactuca* overlaps (38-86 μm) with that recorded for *Enteromorpha linza* (38-95 μm). The neat long rows of cells in surface view were only observed in *E. flexuosa*. Most of the specimens in Table 3.1.1., only had curved or short rows in part or none.

Ulva nematoidea and *U. fasciata* are very similar in many characteristics but the former has somewhat bullet shaped cells in transverse section and cells may be in short rows in surface view.

The number of pyrenoids is very variable. It is normally one in *Enteromorpha* species but up to three were observed in *E. compressa* and *flexuosa*, *E. linza* topped the genus with up to 4 pyrenoids. Among *Ulva* species, *U. uncialis* and *U. rhacodes* were both shown to have a single pyrenoid whereas this character varied considerably among other species.

Blidingia minima was smaller, in every respect, than *Enteromorpha*. The tubes are less than 5 centimetres tall, and in fact from the sampled population none of the thalli exceeded 2 cm. Both in surface view and transverse section, the cells were much smaller than those recorded for *Enteromorpha* species (see Table 3.1.1.).

Table 3.1.1 presents the morphological, anatomical and cytological characters that were used to identify the species. Most of the identifications agreed with those of Stegenga *et al.* (1997), except in cases like *Enteromorpha intestinalis*, in which we found branched forms (LK 42), but in which Stegenga *et al.* (1997) do not note branching. Among other authors Bliding (1963) and Burrows (1991) observed branched forms of specimens identified as *E. intestinalis*.

Table 3.1.1. Descriptions of the investigated specimens

Species name	Thallus morphology	Cell shape (T/S)	Cell shape (S/V)	Cell size (T/S) μm	Cell size (S/V) μm	Thallus thickness (μm)	Cell arrangement	No. o Pyrenoid
B. minima	Tufty hollow tubes, conical	Rounded-rectangular	Rounded-polygonal	(4-6) X (6-8)	(2-4) X (4 X 8)	9.5	None	?
E. compressa	Tubular or partially compressed, very thin branches, constricted at nodes	Rectangular, rounde angular	Irregular	(6-10) X (15-27)	?	29-38	None	1 - (2-3)
E. flexuosa	Compressed, ribbony shaped, branched,		Rectangular-squarish				In long rows	Up to 3
E. intestinalis	Tubular or partially compressed, wrinkled surface, (un)branched, slender	Rectangular	Rectangular-polygonal	(6-12) X (12-25)	(6-13) X (10-17)	38-42	None	1 (-2)
E. linza	Single blade, undulate blade, thin, very soft, unbranched, compressed, crumpled surface, hollow stipe	Rounded-rectangular, rectangular-squarish	Rounded-rectangular, Irregular	(4-15) X (13-32)	(4-13) X (6-23)	38-95	In short or long rows	1- (2-4)
U. capensis	Dentate (double), porous, wrinkled around perforations, undulate, thick lamina, branched, tough basally, ovate, dull and rough, lanceolate, dark patches	Rectangular (rounded angular) slender – bullet spindle shaped squarish-roundish,	Rectangular-rounded, polygonal or irregular, bean shaped and well paired	(8-17) X (27-67)	(4-17) X (8-23)	72-209	Curved rows in part	1-4 (-5)
U. fasciata	Undulate, pale rigid midrib, strap shaped, ribbony, tufted at base, branched to the base	Rectangular-rounded angular, cylindrical conical shaped slender,	Rounded-rectangular, Irregular-polygonal, squarish	(6-25) X (15-46)	(6-15) X (8-21)	57-140	None	1-2 -(3-4)
U. lactuca	Porous, thin, undulate, (un)branched, floating unattached thalli form flat sheets, rough, tougher basally	Rounded t rectangular	Rectangular, rounded-polygonal	(8-15) X (15-25)	(6-15) X (10-21)	38-86	short rows in part	1 -2 (-3)

Table 3.1.1. (*continued*)

U. nematoidea	Midrib, undulate, branched	Rectangular –bullet shaped, rounded angular, slender	Rectangular-squared, rounded-polygonal	(13-19) X (25-40)	(10-19) X (11-36)	67-129	Short rows in part	1 - 3 (-4)
U. rhacodes	Tufty, dentate, epiphytic	Rounded						1
U. rigida	Thick, consistency firm, tufty at the base, incised, entire margins, smooth and shiny, flat sheet, perforated,	Rectangular (rounded angular) –slender	Rectangular-rounded, polygonal, irregular, bean shaped well paired	(6-27) X (21-53)	(6-21) X (10-29)	57-247	Short rows to none	1-3 (-4)
U. uncialis	Rosettes, tough, entire, diminutive 1.5 cm high and fertile	Rectangular	Irregular	(10-15) X (30-36)	(8-13) X (13-17)	82-105	Curved rows in part	1
Ulva sp	Undulate, wrinkled around perforations	Rectangular –rounded	Polygonal, round-squared	(13-19) X (21-27)	(10-17) X (13-21)	53-61	In rows	(1) - 3

Figure 3.1.1: The main thallus is that of *Ulva fasciata* (LK 48), and epiphytic on it is *U. rhacodes*. The arrow indicates a small portion of U. rhacodes that has been isolated from the epiphytic tufts. The specimens came from Dalebrook, False Bay, Cape Town.

Figure 3.1.2: *Ulva nematoidea* (LK 8) from Mile 4 at Swakopmund, Namibia

Figure 3.1.3: *Ulva lactuca* (LK 53) from Simonstown harbour, Cape Town.

Figure 3.1.4: *Ulva rigida* (LK 46) collected from Dalebrook, False Bay, Cape Town.

Figure 3.1.5: *Ulva capensis* (LK 26) collected from Bloubergstrand.

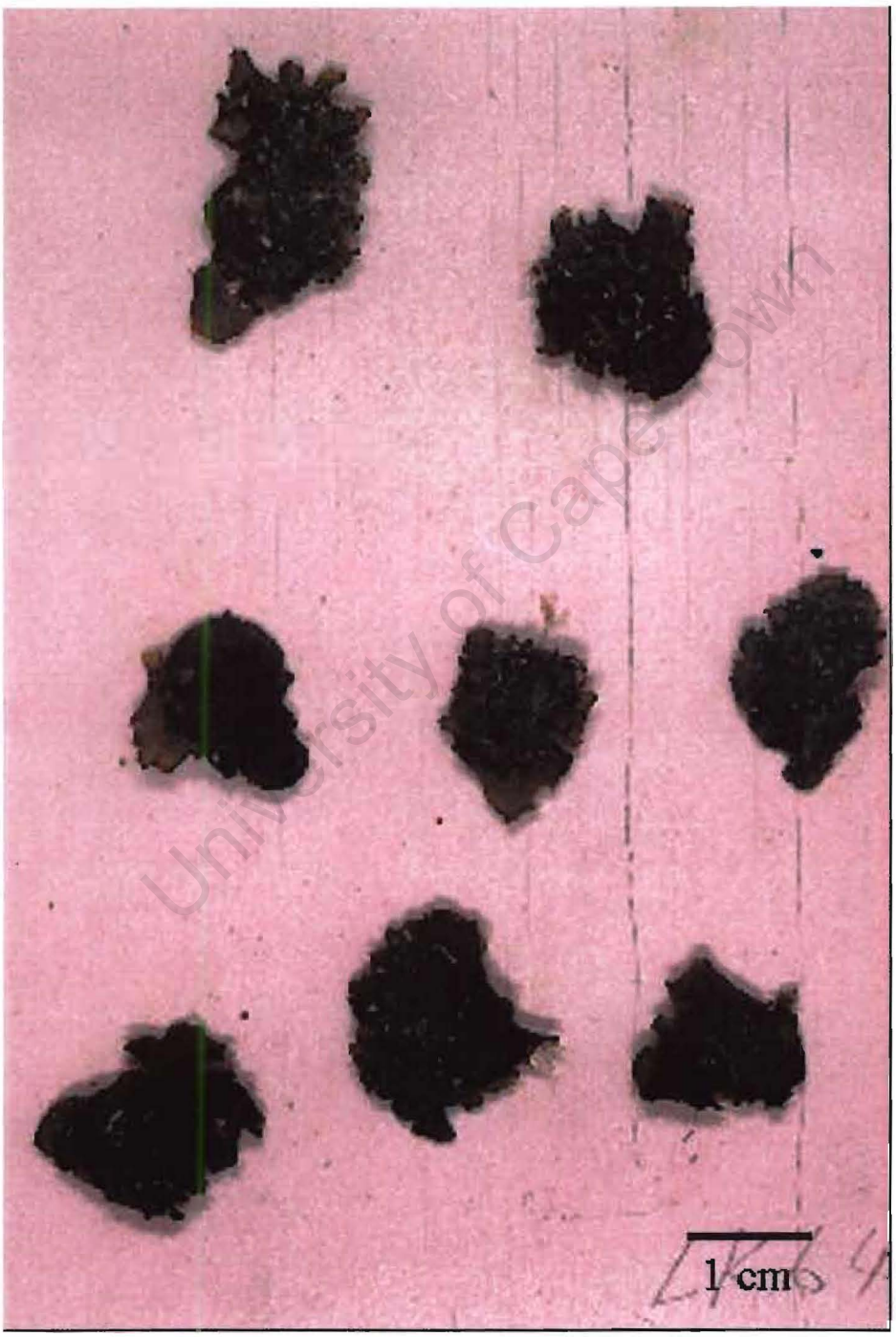

Figure 3.1.6: *Ulva uncialis* (LK 64) collected from Shelly Beach, Still Bay.

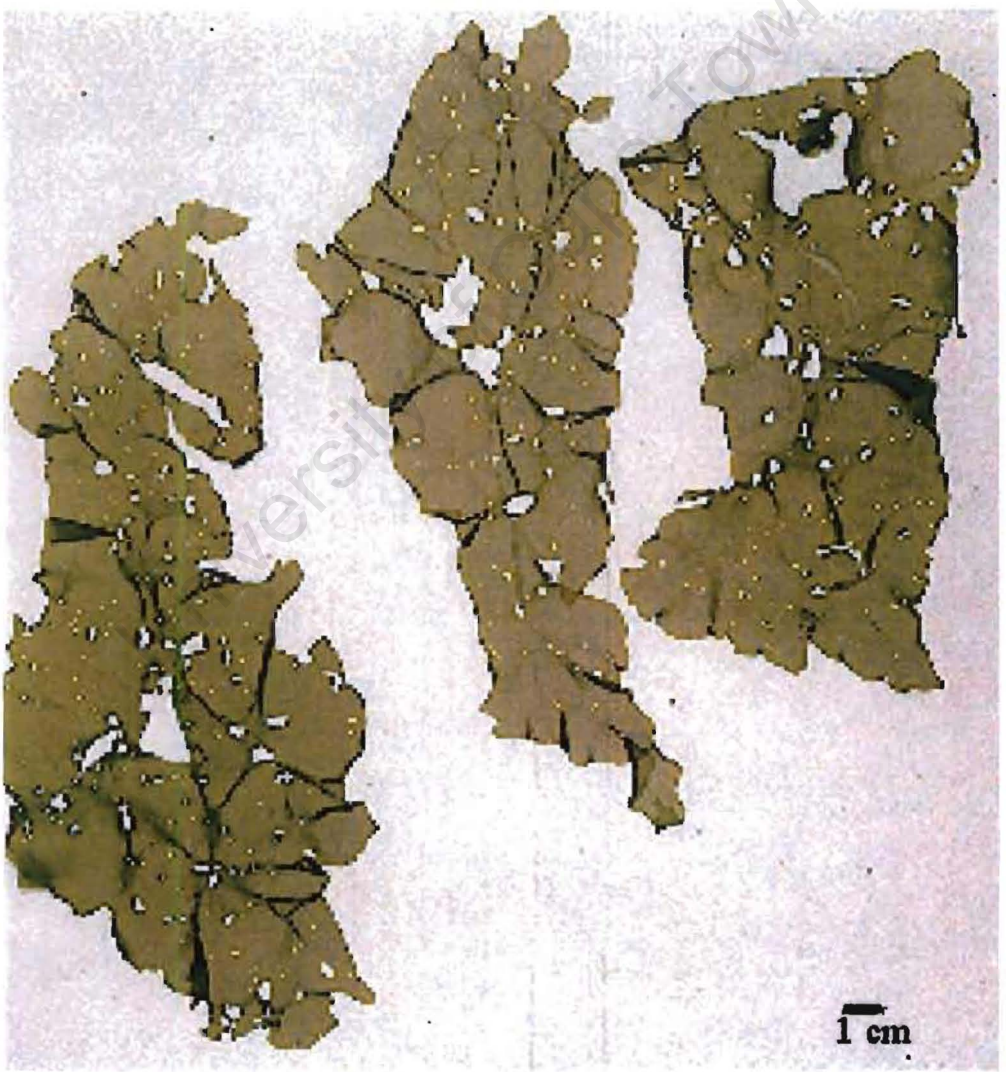

Figure 3.1.7: *Ulva* sp. (LK 5) collected from the I&J Abalone farm, Gansbaai.

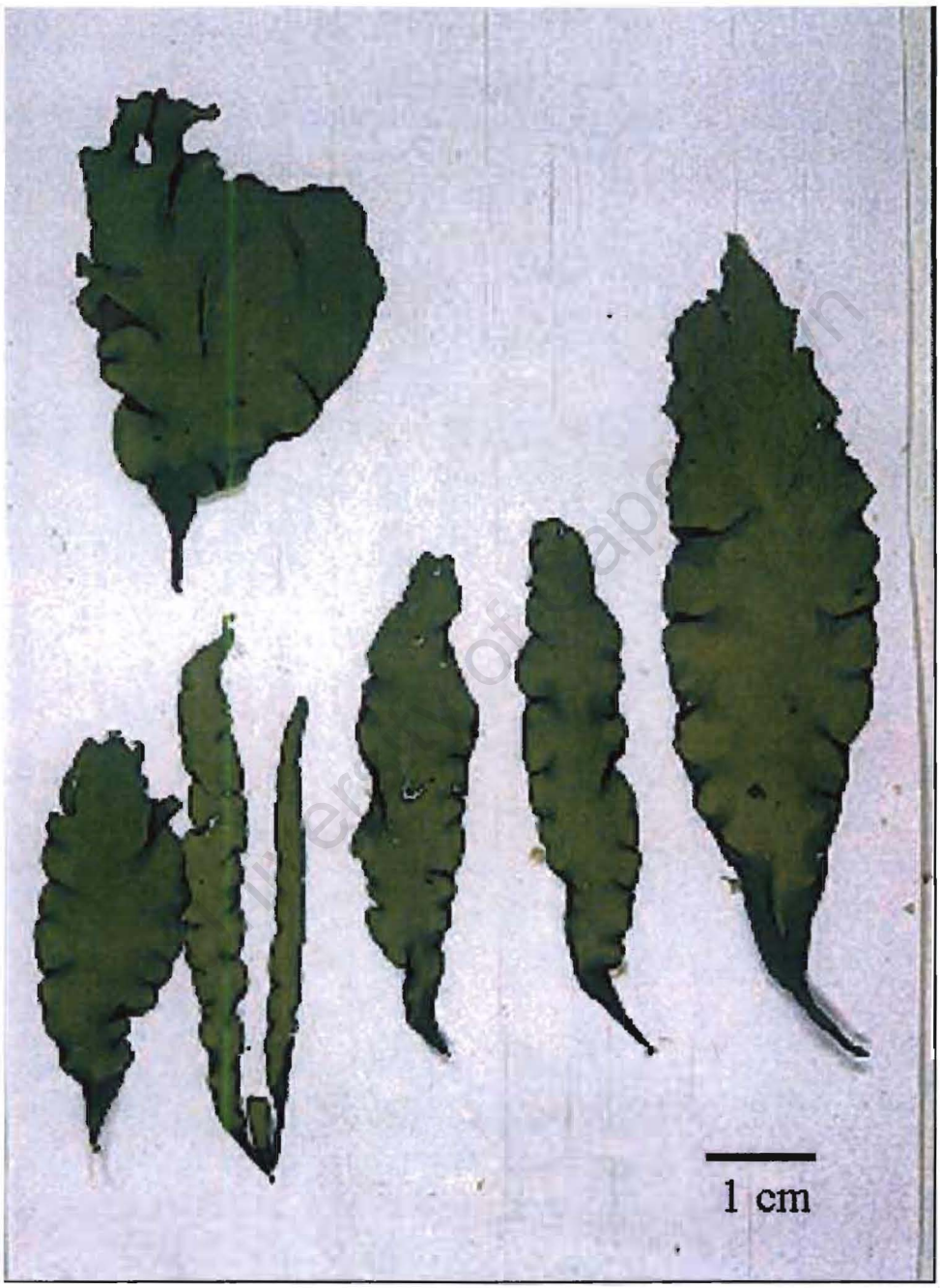

Figure 3.1.8: *Enteromorpha linza* (LK 50) collected from Dalebrook, False Bay, Cape Town.

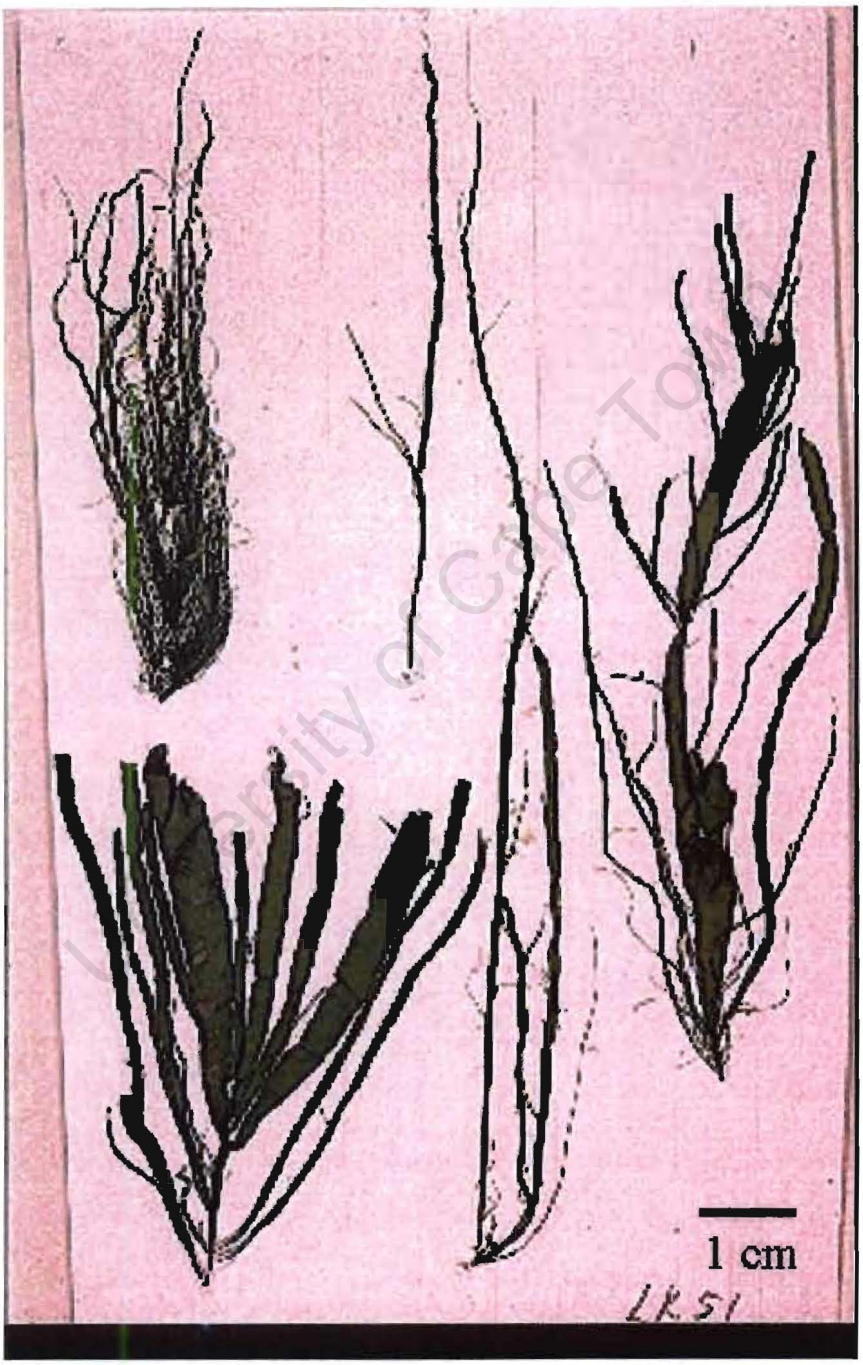

Figure 3.1.9: *Enteromorpha compressa* (LK 51) collected from Dalebrook, False Bay, Cape Town.

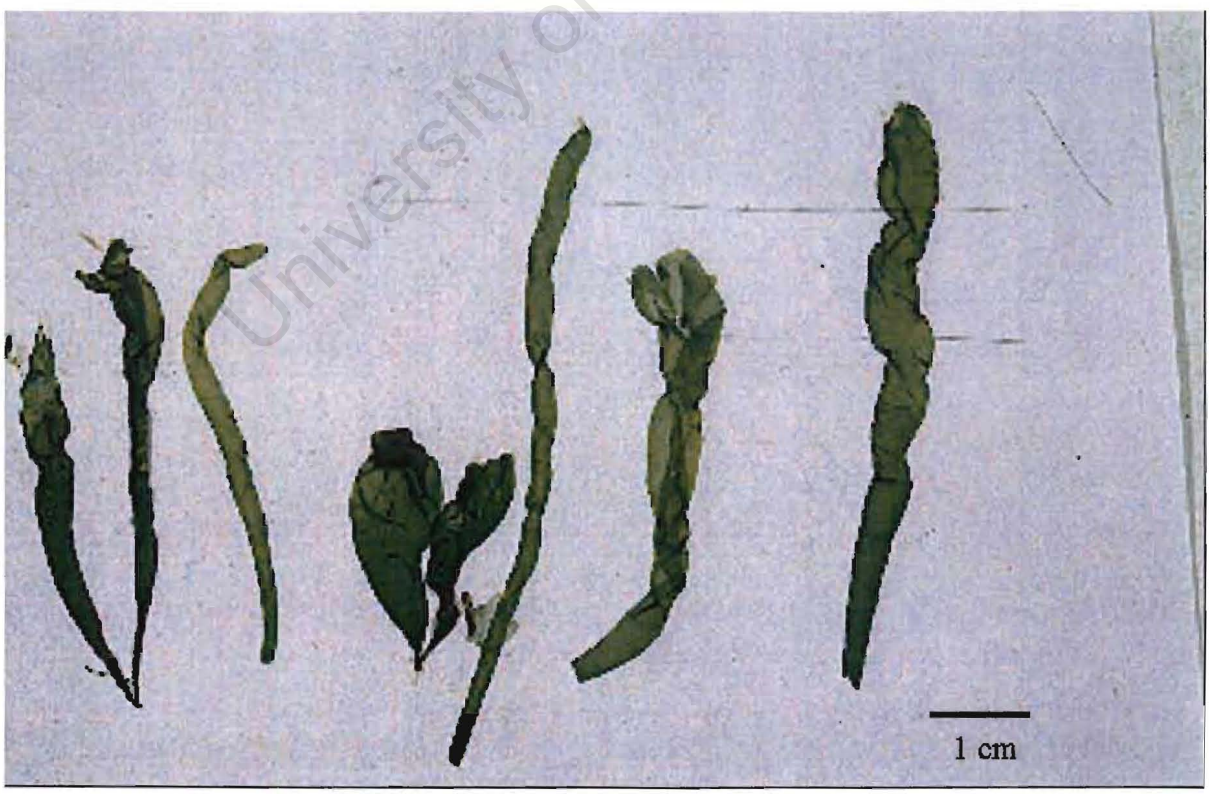

Figure 3.1.10: *Enteromorpha intestinalis* (LK 42) collected from Seapoint, Cape Town

Figure 3.1.11: *Blidingia minima* (LK 63) collected from Jongensfontein, Still Bay.

3.2 Molecular phylogenetic analysis

A total of 49 sequences were obtained, representing seven *Ulva* species, four *Enteromorpha* species and a single *Blidingia* species. The difficulty in sequencing these species was possibly caused by the presence of cellulose material in the extract. Where the extraction succeeded there was still a problem of the possible presence of epiphytic DNA in the extract. This was indeed the case *U. nematoidea*, in which we sequenced a Hydrozoan species, discovered after a blast search to verify the origin of the sequence in GenBank.

3.2.1. Phylogenetic analysis of the complete data set (Ulva and Enteromorpha)

Under equal weights, 16,383 trees were retained (L=328, CI=0.7835, RI=0.9396) and the strict consensus tree (Figure 3.2.1) produced two major clades. The first (A) consists primarily of *Ulva* species and is supported by a fairly high bootstrap value (75%), while the *Enteromorpha* species mostly fall into the unsupported second clade (B), which should currently not be referred as a clade as there is no support. These clades are together supported by a 94% bootstrap value, supporting the exclusion of *Blidingia* from the *Ulva/Enteromorpha* clade. The *U. rigida/capensis* sequences produced a polytomy, with no division into separate species.

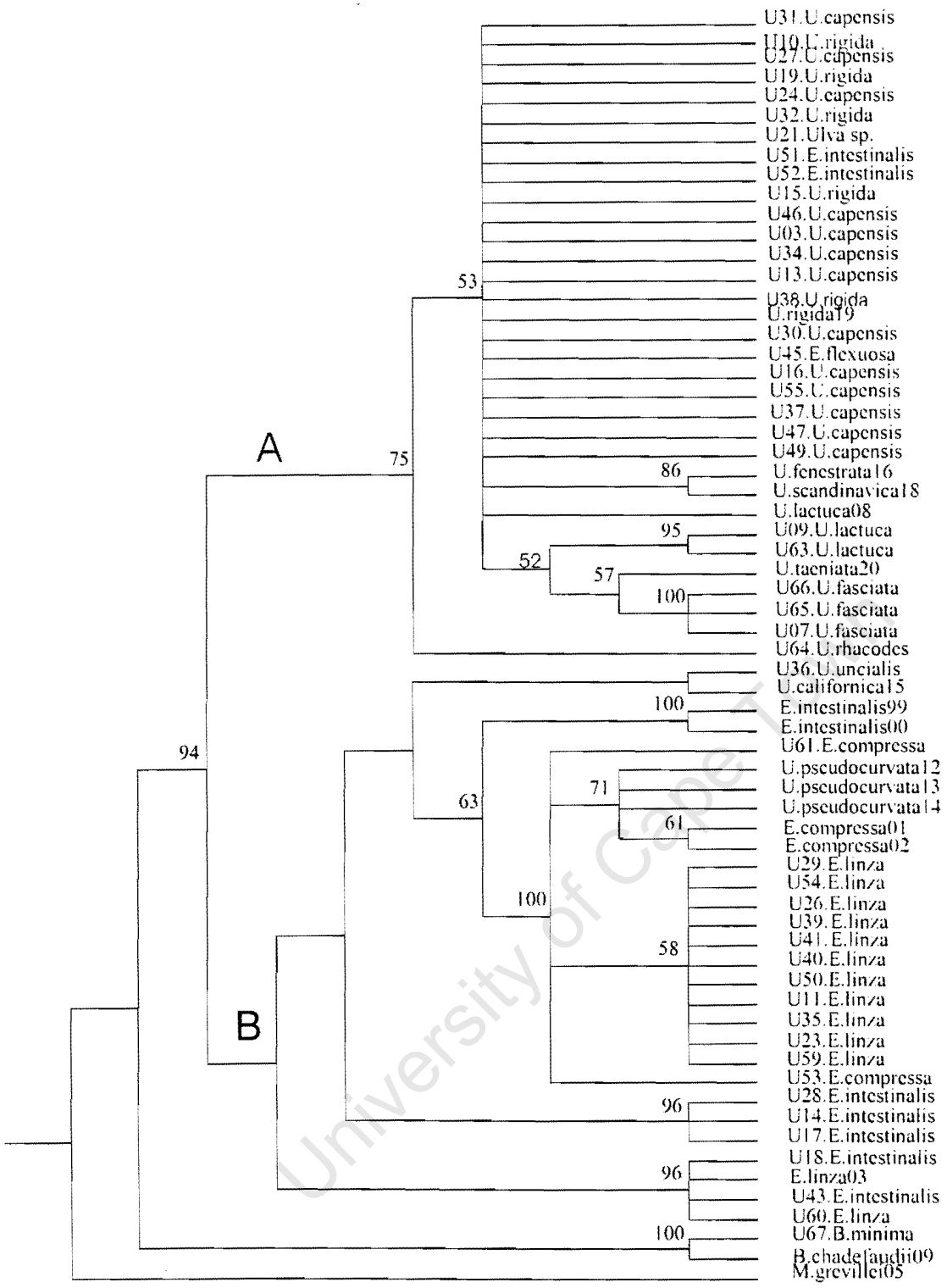

Fig. 3.2.1: A strict consensus tree of 16,383 most parsimonious trees (L=328, CI=0.7835, RI=0.9396) generated in a heuristic search under maximum parsimony of all local taxa and those obtained from GenBank. The relationships are based on the ITS1, 5.8S and ITS2 sequence data. The numerals on the branches the bootstrap percentages (BP) from 100 replicates. **A** indicates the "*Ulva*" clade and **B** the "*Enteromorpha*" clade. *M. grevillei* is the outgroup. Note: local samples are preceded by a code (e.g. U03 in U03.U.capensis), which was the analysis code, whereas the GenBank samples are suffixed with a number (e.g. 19 in U.rigida19), which represents the last two digits of the GenBank accession number.

All the samples corresponding to the description of *U. rigida* and *U. capensis*, plus the unidentified *Ulva* sample (U21) produced a polytomy. Interestingly though, two samples identified as *Enteromorpha intestinalis*, plus a sample that corresponded to *E. flexuosa* (Stegenga *et al.*, 1997) were grouped with this clade. All three were collected from Seapoint, Cape Town. The other *Ulva* species were better resolved apart from "U. lactuca08", whose identity is somewhat dubious. According to GenBank the sequence corresponded to *U. lactuca*, whereas Tan *et al.* (1999), assigned the same accession number to *U. rigida*. Unsuccessful attempts were made to clarify the issue with the authors, but it is however most certain that this species is definitely *U. rigida*. Closely associated with this grouping are other *Ulva* species such as the clade of *U. fenestrata* and *U. scandinavica*, which are very well supported with a bootstrap value of 88%. Also associated with the *U. capensis/rigida* clade was the poorly supported clade (52%) of *U. lactuca*, *U. fasciata* and *U. taeniata*. The *U. lactuca* clade is well supported with a bootstrap of 95%. The clade containing the latter two is poorly supported (57%), but the terminal node encompassing the three isolates of *U. fasciata* is well supported with a very high bootstrap of 100%.

Within the *Enteromorpha* clade, the samples from supposedly different species are completely mixed up. Accessions of the type species of the genus *Enteromorpha*, *E. intestinalis*, do not form a monophyletic clade, instead they are distributed between three separate lineages. The GenBank accessions of *E. intestinalis*, which represent the type specimen of the species since they are of European origin where the type was collected from, form a well-supported clade (100%). This clade in turn, also forms a sister-group (63%) with a clade, which is also strongly supported (100%), composed of *E. compressa* (both from GenBank and local) *U. pseudocurvata*, and many local specimens of *E. linza*. The clade made up of *U. pseudocurvata* and *E. compressa* is linked at a bootstrap percentage of 71%. The clade containing

the European (GenBank) *E. compressa* has a bootstrap support of 61%. Another lineage of *E. intestinalis* (U14, U17 and U28), which is well supported (96%), is sister to the *Enteromorpha* clade discussed above, which also incorporates *Ulva* species (*U. uncialis* and *U. californica*). This clade, joined at **B** is in turn sister to another yet well-supported clade (96%) consisting of two local isolates of *E. intestinalis* (U.18 and U.43) and two *E. linza*, one (E.linza03) from GenBank, and the other (U60) one being local. The *Enteromorpha* clade will be investigated further at a later stage. *Blidingia minima* and *B. chadefaudii* form a well supported clade (100%), which is a sister-group to the clade containing all *Ulva* and *Enteromorpha* species. But as mentioned earlier, the sister relationship of *Blidingia* to *Ulva/Enteromorpha* is not supported at all, the latter grouping having a bootstrap of 94%.

3.2.2. Analysis of the Ulva capensis/rigida grouping

The strict consensus tree of the 166994 most parsimonious tree (L=246, CI=0,915, RI=0.727) presented in Fig. 3.2.2. shows that the *Ulva capensis/rigida* clade is well resolved and the grouping is moderately well supported (77%). The included *Ulva* species were closely associated with the *capensis/rigida* clade with a high bootstrap support of 99%. *U. lactuca* is sister (58%) to clade containing *U. fasciata* and *U. taeniata* and the latter clade is also well-supported(69%). Apart from the *E. intestinalis* and *E. flexuosa* samples that were grouped within the *U. capensis/rigida*, the only other *Enteromorpha* species included, *E. linza*, was sister to the clade containing the *Ulva* species.

Similar results were also obtained when the polytomous clade was explored using TCS analysis (Fig. 3.2.3). The graph produced five haplotypes, of which the one with the highest probability (the box) consisted of 21 isolates composed of a combination of *U. rigida* (5) and *U. capensis* (9), and among these were the *Enteromorpha* (3) specimens and the odd other *Ulva* sp. The other

four haplotypes each represented a single isolate and differ from the common haplotype by a single base change each. Most accessions are identical at this locus.

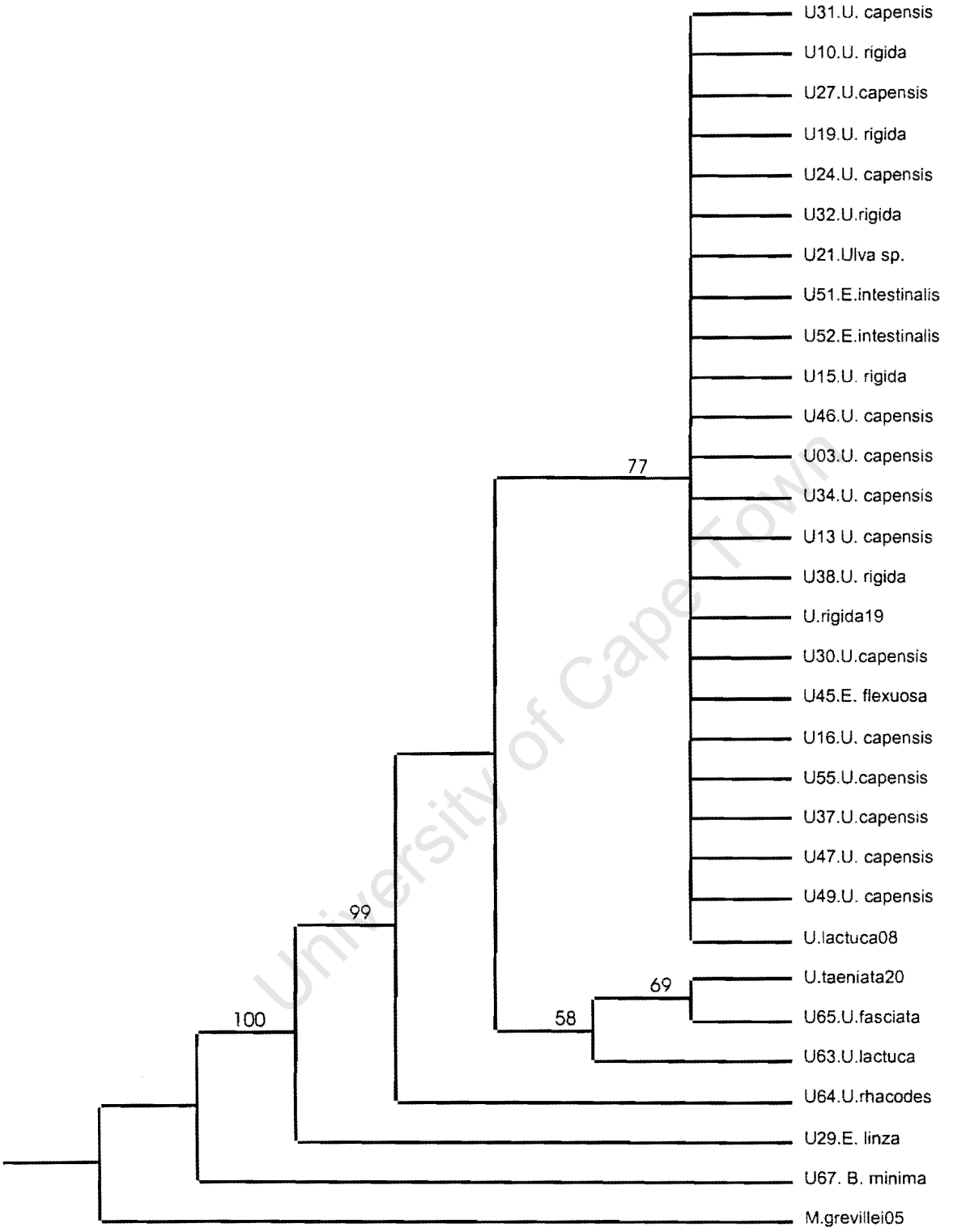

Fig. 3.2.2. The strict consensus of 1000 most parsimonious trees (L=246, CI=0.915, RI=0.727) generated in a heuristic search under maximum parsimony. The relationships are based on the ITS1, 5.8S and ITS2 sequence data. The numbers on the branches indicate the percentage bootstrap support from a hundred replicates. *M. grevillei* is the outgroup.

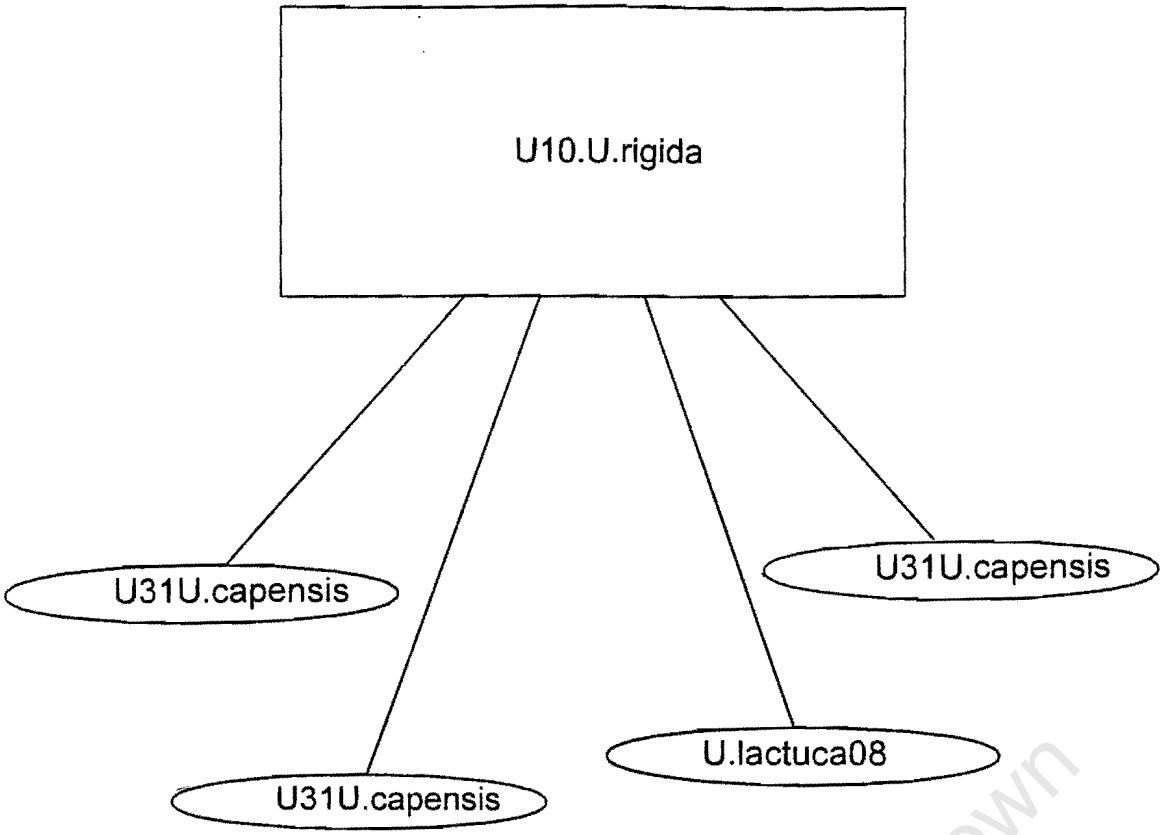

Fig. 3.2.3. The TCS graph showing the relationship among the *Ulva capensis/rigida* haplotypes. Note that the U10.U.rigida (the box) consists of 21 isolates namely; *U. rigida* (5), *U. capensis* (9), *E. intestinalis* (2) *E. flexuosa* (1) and *Ulva* sp (1).

3.2.3. Analysis of the *Enteromorpha* clade

Presented in Fig. 3.2.4 is the strict consensus tree (L=366, CI=0.803, RI=0.845) of the 166994 most parsimonious trees. The ingroup is well supported (99%) and it is divided into two main clades, the upper one, which is unsupported by the bootstrap, is composed primarily of *Enteromorpha intestinalis* specimens, whereas the lower one, which is strongly supported (97%), is made up of *Enteromorpha linza, E. compressa* and *U. pseudocurvata*. Within the *E. intestinalis* grouping, the terminal nodes are generally well supported, including the clade (99%) composed of two *E. intestinalis* (U18 and U43) and two *E. linza* (U60 and E.linza03) accessions. Two *Ulva* representatives (*U. rhacodes* and *U. fasciata*) form a well-supported (84%) clade.

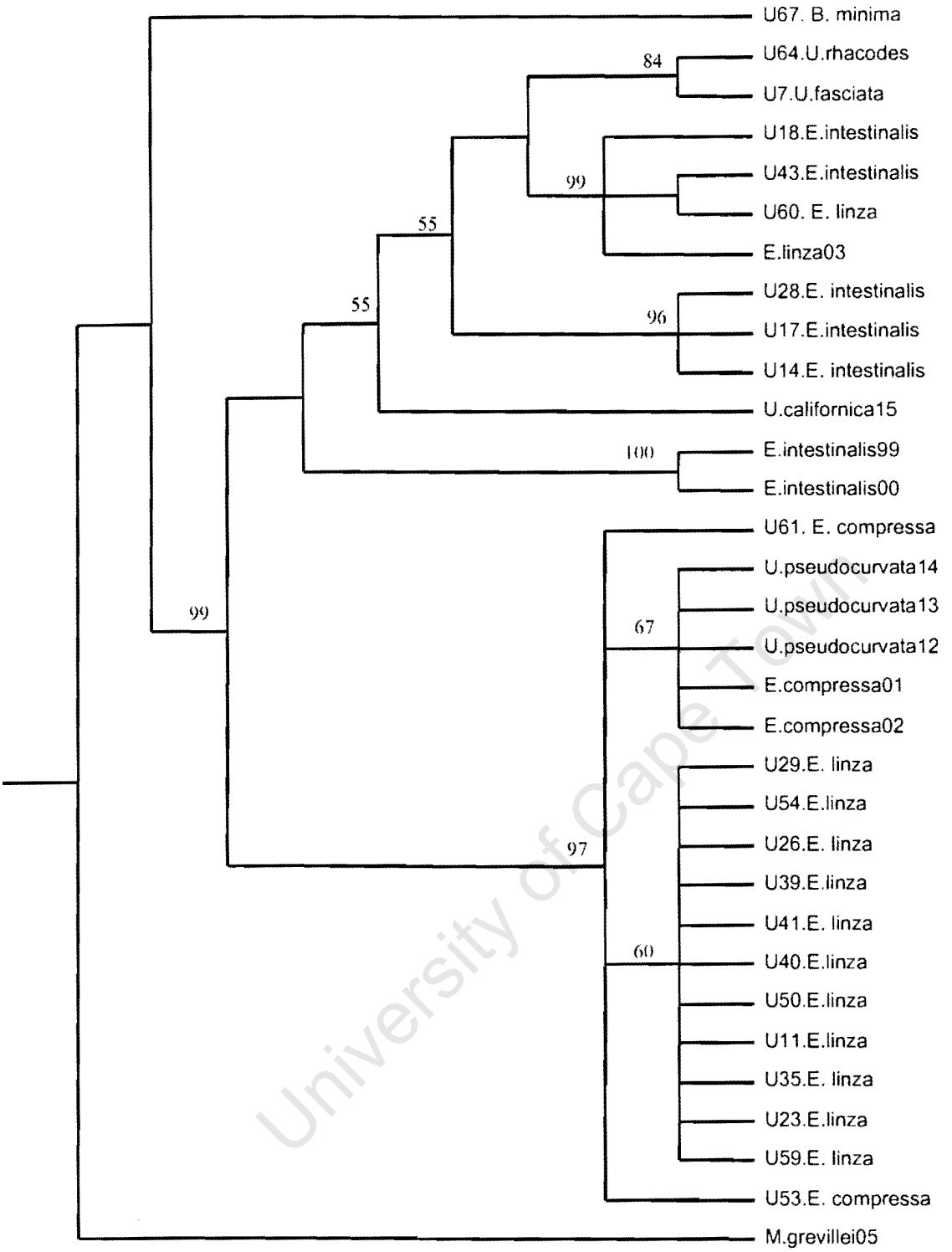

Fig. 3.2.4. The strict consensus tree (L=366, CI=0.803, RI=0.845) of the 166994 most parsimonious trees, showing the relationships among the *Enteromorpha* specimens. The relationships are based on the ITS1, 5.8S and ITS2 sequence data. Also included are *Ulva species* that were nested within (*U. pseudocurvata* and *U. californica*), as well as *U. rhacodes*, *U. fasciata* and *B. minima* as outgroup taxa. The tree is rooted on *M. grevillei*.

As in the earlier results (Fig. 3.2.1.), the isolates of *U. pseudocurvata* and *E. compressa* from Tan *et al.* (1999) form a clade that is moderately well supported (67%). What is worth noting here is that *Ulva* spp. are nested within the *Enteromorpha* clade although there is poor support for this inclusion. A particularly interesting observation is that of *U. pseudocurvata*.

surface and they are obviously, as Dion *et al.* (1998) put it, often impossible to apply to dried material.

All investigated thalli were found to have rectangular cells in transverse section. The observation also applies to *U. capensis* and *U. lactuca*, which normally bear spindle or bullet shaped and rounded cells respectively, when the upper section is examined. Our finding is in agreement with many other authors such as Phillips (1988) and Bliding (1968) who found that cell shape in transverse section is more variable in the mid section than in the basal section, and highly variable in the upper/marginal section. In surface view cell shape is potentially useful but the same rounded to rectangular shape was observed in most of the specimens except in specimens identified as *E. compressa* and in *U. uncialis* where the cell shape is irregular. In specimens identified as *U. capensis* it was noted that many cells were rather bean shaped. Cell size was found to be very variable even within a single specimen. *Enteromorpha* species generally have small sized cells, except for *E. linza*, which had cells with magnitudes in the range of those recorded for *U. lactuca*. The only specimen that could confidently be separated from others based on cell size is *B. minima*, which has very small cells, the highest being 8 μm compared to others such as *U. capensis* which can reach heights of up to 67 μm. As one would expect, cell size especially in transverse section is closely correlated with thallus thickness. Thallus thickness is fairly reliable as a diagnostic character. For instance among *Ulva* species, *U. capensis* and *U. rigida* are generally very thick (up to 207 μm and 247 μm, respectively) compared to others such as *U. fasciata* and *U. nematoidea*, which are thick along the 'midrib' or else only in the basal section. A problem associated with the cell size character is that it may vary with age (Bliding, 1968; Coat *et al.* 1998), ploidy level (Coat *et al.* 1998), season (Titlyanov *et al.* 1975; Phillips 1988) and wave exposure (Tanner, 1986, Phillips, 1988).

The cell arrangement in surface view was considered by Bliding (1968) as being characteristic for a species. In this study it was shown that apart from specimens identified as *E. flexuosa* and *E. linza*, most only had short rows in parts whereas others such *U. fasciata, E. intestinalis, E. compressa* and *Blidingia minima* were not arranged in any particular order. The same pattern of results has been reported by Hoeksema and Hoek (1983) and Phillips (1988).

Bliding (1968) as well as some other workers (e.g. Koeman and Hoek, 1981) considered pyrenoid number to be taxonomically informative. Kapraun (1970) however considered it to be too variable for taxonomic use and this variation was also reported by Phillips (1988). In this study, it was found that most species have at least two pyrenoids. Among *Enteromorpha* species, most specimens had one whereas within *Ulva* the number was found to be very variable. The only species that consistently had one pyrenoid were *U. rhacodes* and *U. uncialis*. This study is therefore in agreement with Kapraun and Phillips, but indeed the character can be used successfully in certain species as shown above.

The results based on analysis of nuclear DNA sequence data, adds to the growing body of criticism against defining taxa solely on the basis of morphological data. This is ofcourse only true if DNA data are assumed to be telling the true story. However, the biggest problem with these algae, which has already been addressed above, is the morphological plasticity, a problem common to all members of the Ulvaceae (Blomster, *et al.*, 1999).

The main feature presented by the analysis is the fact that, neither *Ulva* nor *Enteromorpha* is monophyletic (Fig. 3.2.1.). However, this study does not fully support the idea of collapsing the two genera into one as expressed by some

studies (e.g. Blomster *et al.*, 1999; Tan *et al.* 1999; Woolcott and King, 1999) and this is clearly shown in Figure 3.2.1. The phylogenetic tree (Fig. 3.2.1.) suggests two separate lineages; one very *Ulva*-like and the other one mainly *Enteromorpha*-like. Therefore the idea of collapsing the two genera is premature and the possibility of finding new synapomorphies remains. It is however noted here that there is a lot of evidence pointing towards a merger of the two genera.

In our analysis, two species of *Enteromorpha*, *E. intestinalis* (two isolates, see Fig. 3.1.9.) and *E. flexuosa* (all from Seapoint, Cape Town) were grouped within the *Ulva* clade (Fig. 3.2.1 & 3.2.2). These samples were indeed small/young. From the developmental biology of *Ulva* and *Enteromorpha* species, it is known that the two genera share the same developmental pattern, and all *Ulva* species pass through a tubular *Enteromorpha*-stage. When the plant reaches a few millimetres in height, the tubular thallus becomes compressed, causing the plant to become distromatic (*Ulva*-like) (Bliding, 1968). It is therefore possible that the *Enteromorpha* accessions included within the *Ulva* clade could well be immature specimens of what are generally considered to be *Ulva* species.

An environmentally induced morphological variation could also have led to the differentiation of the *Ulva* sp. (Fig. 3.1.11) that was grouped within the *Ulva capensis/rigida* clade. The plant was collected from the I&J abalone farm where it was grown in tumble culture. It is possible that this specimen could have been altered by the conditions in the tanks because no such forms have been recorded from natural populations.

The inclusion of *Enteromorpha* species within the *Ulva* clade was mirrored by the grouping of *U. pseudocurvata* within a well supported clade (100%) composed of *E. compressa*, and *E linza* (Fig. 3.2.2.). Our results are similar to those obtained by Tan *et al.* (1999). According to Tan *et al.*, *U. pseudocurvata*

samples had a typical *Ulva* morphology of simple distromatic blades without cavities. The results are therefore very peculiar since local *E. compressa* specimens (Fig. 3.1.8.) and those cited by Tan *et al.* are branched. However, this clade with 100% support from U61.E.compressa to U53.E.compressa, is almost certainly all *E. compressa* (including *U. pseudocurvata*), although it is possible that all the *E. linza* sequences represent a separate entity - perhaps *E. atroviridis* (Levring) Wynne, another *Enteromorpha* species that is endemic to the South west coast of Southern Africa. *E. atroviridis* was not collected during this study, but it is closely related to *E. linza* and they occupy overlapping ranges (Stegenga *et al.* 1997). The association of *E. linza* with *E. compressa* is also against Bliding's groupings. Bliding (1963) placed *E. linza* in a group of its own, the Linza group, whereas *E. compressa* and *E. intestinalis* were placed in the Intestinalis group.

The '*E. intestinalis*' clade (U28, U14 and U17) with 96% support is probably the most interesting of all because it is hard to make anything out of it, rather then saying that it is probably a sibling species in the genus *Enteromorpha*.

The clade (Fig. 3.2.1 and Fig. 3.2.4) containing a combination of *E. linza* (U60 and E.linza03), and *E. intestinalis* (U18 and U43) is very interesting. The group is very well supported (96%) and far removed from the other members of the same species. The most feasible explanation for this result is that the group (U60, U18, U43 and E.linza03) represents the true *E. linza*, especially since the only European *E. linza* used in this study is within this clade.

The inclusion of *U. californica* and *U. uncialis* in the *Enteromorpha* clade is unexpected, and this requires further investigation to determine why this is the case.

Also closely associated is the local species of *Blidingia minima* and the European species *Blidingia chadefaudii*, which form a well-supported clade (100%). This finding is very interesting since Tan *et al* (1999) also included both a *B. chadefaudii* (from Northern Ireland) and *B. minima* (Scotland) in their analysis, but the relationship between the two was not supported. Woolcott *et al*. (2000), working on the Japanese *B. minima* and *B. chadefaudii*, found that the two species were conspecific and suggested a merger of the two. *B. minima* as identified in this study had cellular dimensions that corresponded well with those of Bliding (1968), Burrows (1991) and Woolcott *et al*. (2000). According to Bliding (1963) and Burrows (1991) *Blidingia chadefaudii* differs from *B. minima* in having thickened thallus walls, and elongate cells that may be up to 30 μm long or more. This information justifies our identification, but we cannot draw a conclusion on the relationship between the two as yet, because the description of the material sequenced by Tan *et al*. (1999) is not available. Caution is being taken here because Burrows (1991) pointed out that the two species are easily confused and they are sometimes sympatric.

4.1.1. Is *Ulva capensis* synonymous with *U. rigida*?

Papenfuss (1940) noted the fact that many authors regarded *U. capensis* [*Ulva capensis*, Areschoug 1851: 15-16] to be synonymous with *U. rigida* [*Ulva rigida*. C. Agardh, 1823 (1822-1823): 410-411]. When seen from the vertical section of the thallus, *U. capensis* has cells that are much longer than those of *U. rigida*. Papenfuss therefore suggested that the two were distinct species. The characters (Table 3.1.1) used to distinguish these two species are that *U. capensis* is dentate and has spindle or bullet shaped cells in transverse section. *U. rigida* has rectangular cells, entire margins. The cells in surface view are also often described as being in long rows, but this is not always the case. Stegenga *et al*. (1997), indicated that *U. rigida* may also possess minute teeth,

something not observed during the course of this study. However, it appears that all the characters that describe local species of *U. capensis* correspond to the European (Bliding, 1968; Hoeksema and Hoek, 1983), Australian (Womersley, 1984) and New Zealand (Adams, 1994) *U. rigida*.

Our phylogenetic analysis which is the first such to probe the relationship between *U. rigida* and *U. capensis*, suggests that there is no difference between the two according to ITS. This was clearly shown by maximum parsimony (Fig. 3.2.1) and TCS analysis (Fig. 3.2.3). According to TCS analysis, these species are not even separate haplotypes of a diverse species. The two occur in overlapping ranges, *U. capensis* occurring from Namibia (west coast) to Cape Agulhas (Joska, 1992; Stegenga *et al*. 1997; see Fig. 2.1.1.) while *U. rigida* occurs form the Cape Peninsula-eastwards (Joska, 1992; Stegenga *et al*. 1997), based on existing records. However, in this study samples corresponding to *U. rigida* were also collected from Yzerfontein and Swakopmund, Namibia, which are both west coast sites. Therefore, if the anatomical differences are environmentally induced, then such factors are probably local in effect. The specimen collected from the I&J farm could also represent yet another form of the polymorphic species represented by *U. rigida* and *U. capensis*.

4.1.2. Does *U. uncialis* represent a distinct lineage?

According to Stegenga *et al*. (1997), *U. uncialis* [(Kützing) Montagne, 1850: 246] closely resembles *U. rigida* [C. Agardh, 1823 (1822-1823): 410-411]. It is tufty and cuneate, and first impressions are that it may be an *U. rigida* that has been reduced in size due to grazing or desiccation. The reduction in size could also be due to wave action since this form generally grows near the high water mark. Silva et al. (1996) however challenged the legitimacy of applying the name *U. uncialis* in the sense of Stegenga *et al* (1997). According to Silva *et al*., the name *Phycoseris uncialis* was first used

without a description (Kützing, 1843: 297), with the Cape of Good Hope and Peru being mentioned as origin and Suhr's manuscript name *U. uncialis* being cited in synonymy. The South African collection cited by Kützing was said to come from Table Bay (Drege, 1843: 111). Papenfuss (notes) confirmed that Suhr's specimens of *U. uncialis* came from Table Bay as well as from the "Kaffernkuste". On the other side, Areschoug, when establishing *U. capensis*, cited "*Ulva uncialis* v. Suhr. - Drege Docum. P. 111." and stated that specimens distributed under this name were juveniles of *U. capensis*. The same sample (Drege's material) was therefore assigned two different names. Since Kützing (1849: 475) had already proposed a new species for Drege's material (*P. uncialis*), *U. capensis* was therefore thought to be redundant and consequently illegitimate in accordance with Art. 52.1. Therefore if the species now referred to as *U. uncialis* exists and is distinct from *U. rigida* (including *U. capensis*) then it needs to be assigned a new name.

The phylogenetic analysis of this study suggests that *U. uncialis sensu* Stegenga *et al.* (1997) does exist and is distinct from *U. rigida* (Fig. 3.2.1). Instead its closest relative, among those species included in the analysis, is *U. californica* Wille, from Oregon, U.S.A. *U. californica* has been described by Tanner (1986) as being diminutive, tufted and cuneate. This morphological description fits that of local specimens of *U. uncialis*, or at least the one used in this investigation. Thallus and cell dimensions are generally larger in *U. uncialis*. However, when one takes into account other species such as *U. angusta* Setchell & Gardner and *U. scagelii* Chihara (which Tanner synonymised), cellular dimensions do generally fall within the range of *U. uncialis*. Further work is needed to reach any conclusive results on this, since there was no bootstrap support for this relationship. In addition, our molecular analyses are based on

single specimen of each, and our measurements were only based on a single collection.

4.1.3. The relationship among the linear forms

Although poorly supported, a close association was shown between *U. fasciata* and *U. taeniata*, which are both lanceolate, with ruffled margins. *U. taeniata* has been synonymised with other linear, undulate species such as *U. stenophylla* (Phillips, 1988; Adams, 1994) and *U. nematoidea* (Wynne, 1986). It is a pity that *U. nematoidea* could not be sequenced because it could have given us more evidence on lanceolate species. Our analyses suggest that *U. lactuca* is closely related to *U. fasciata* but the relationship is poorly supported. Phillips (1988) grouped *U. lactuca* among other *Ulva* species that may have a linear thallus under some circumstances. This is hard to observe in local species of *U. lactuca* because they generally occur in bays and harbours, where they normally occur unattached, and as a result lose orientation. Woolcott and King (1999) also found a close relationship between *U. fasciata* and *U. lactuca*.

The results from our molecular data shows that variation in the ITS sequence is not geographically structured. This is shown for instance in the close association of *U. taeniata* from Oregon, U.S.A with the local species of *U. fasciata*. *U. rigida* from Orkney, Scotland is also caught up in the local mixed clade of *U. rigida* and *U. capensis*. Also closely associated is the local species of *Blidingia minima* and the European species of *Blidingia Chadefaudii*, which form a well-supported clade (100%) that is sister to a well-supported (94%) *Enteromorpha/Ulva* clade. This suggests many migrations in historical time.

It is, however, important to note that this study is only based on a single molecular marker, and therefore these

findings needs to be backed up by the use of alternative markers especially those of the chloroplast origin.

Chapter 5: Conclusions

The idea of collapsing the two genera, *Ulva* and *Enteromorpha*, is not well supported by this study. The study has shown that there are definitely two separate lineages, although some species from one genus may be nested within the other.

Our study fully suggests that *Ulva capensis* and *U. rigida* represent a single polymorphic species. This would then imply that *U. capensis* Areschoug, 1851 be placed as a synonym under *U. rigida* C. Agardh, 1823. In turn, the specimens identified under the original description of *U. uncialis* (Kützing) Montagne 1850 should be synonymised under *U. rigida*. This must however be done carefully so as not to synonymise records that were described *sensu* Stegenga *et al.* (1997).

Another interesting conclusion would be the recognition of *U. uncialis sensu* Stegenga *et al.* (1997) as a distinct species. However, following from Silva *et al.* (1996), a new name will have to be assigned. These conclusions are reserved pending further investigation. It is, however, strongly believed that any investigation based on the ITS gene region will arrive at the same conclusion, especially concerning the synonymy of *U. capensis* and *U. rigida*.

Another species that we might lose in this region is probably *E. intestinalis* because this study indicated that what is referred to, as *E. intestinalis* here in Southern Africa does not correspond to the European samples.

The one thing is most conclusive from this study is that the current genera do not work and the two genera, *Enteromorpha* and *Ulva* are not monophyletic, but it is very probable that after further research, more genera could be revealed within the group. A symptom of the problem is that a branched species like *E. compressa* is closely associated with a distromatic

blade such as *U. pseudocurvata*. *Blidingia* is also a genus that must be investigated further in order to determine whether *B. minima and B. chadefaudii are conspecific*, as well as to determine its relationship with the other two genera.

However, the one thing that we have to keep in mind is that "molecular data are not a magic bullet for the species problem, because just like morphological data they can be misleading." Manhart and McCourt (1992) Pp. 735. The exceptional fact about molecular data is that they are more robust (there are only four possible bases) than conventional data.

Chapter 6: Recommendations for future investigations

Subsequent studies will have to include a wider sampling region, preferably the whole southern coastline from Mozambique to the Angolan/Namibian border. This would help reach a more conclusive decision, as it will include a range of environments, which should also help address the problem of morphological variation. On a global scale, there is a need for a collaboration to launch a DNA survey of all known species. This could also be done on a regional basis, after which all the data will be put together (as in GenBank) and then a complete phylogeny will be produced.

Many molecular studies are now moving towards a combined type of analysis, whereby molecular data is combined with morphological and anatomical data. Given more time, this study was going to look at this aspect. Another thing that could be envisaged is the use of multiple gene regions. Chloroplast genes could be useful in this regard.

Chapter 7: References

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Appendix I

The nomenclature of the *Ulva* and *Enteromorpha* species that are common in the region.

Ulva Linnaeus, *nom cons*

Ulva capensis Areschoug

Ulva capensis, Areschoug 1851: 15-16

Areschoug, 1854: 369-370. - Papenfuss, 1940: 4. - Bolton & Stegenga, 1987: 168. Joska, 1992: 30, Pl. 31. Stegenga, Bolton & Anderson, 1997: 91, Pl. 14. Kandjengo. 2000: 10.

Ulva minuta Papenfuss *ined.* - Stephenson, 1948: 297. Isaac, 1953: 63, 71.

Phycoseris lobata africana Kützing, 1849

Phycoseris capensis Grunow, 1867.

Ulva fasciata Delile

Ulva fasciata Delile, 1813-1826: 297, pl. 58: fig. 5 (type locality: Alexandria, Egypt).

Seagrief, 1988: 38, fig. 5:1. - Anderson & Stegenga, 1989: 302. - Bolton & Stegenga, 1990: 237. - Farrell, Critchley & Aken, 1993: 150. - Joska, 1992: 39-47. - Stegenga, Bolton & Anderson, 1997: 92. Kandjengo. 2000: 10. Steyn, 2000: 22-31.

Ulva lactuca Linnaeus

Ulva lactuca Linnaeus, 1753: 1163 (type locality: "in oceano").

Stephenson, Stephenson, & du Toit, 1937: 356, 363, 372. - Eyre, Broekhuysen, & Crichton, 1938: 90, 93, 101. - Eyre & Stephenson, 1938: 33. - Stephenson, Stephenson, & Bright, 1938: 18. - Isaac, 1942: 233. - Stephenson, 1944: 313. -

Stephenson, 1948: 297. - Joska, 1992: 48-53. - Stegenga, Bolton & Anderson, 1997: 93. Kandjengo. 2000: 11

***Ulva rhacodes* (Holmes) Papenfuss**

Enteromorpha rhacodes Holmes, 1894, 89-90 (type locality: Port Alfred, Cape Province, South Africa). Barton, 1896: 193. - Delf & Michell, 1921: 92.

Ulva rhacodes (Holmes) Papenfuss, 1960: 311, figs. 7-9, pl. 6 (including Indian Ocean record). Seagrief, 1988: 38, fig. 5:1. Joska, 1992: 54-60. - Stegenga, Bolton & Anderson, 1997: 95, Pl. 16.

***Ulva rigida*. C. Agardh**

Ulva rigida. C. Agardh, 1823 (1822-1823): 410-411 (lectotype locality: Cadiz, Spain fide Papenfuss, 1960: 305). - Stephenson, 1948: 297. - Isaac, 1953: 63. - Isaac, 1956: 165. - Isaac, 1957: 77. - Isaac, 1958: 20. - Pocock, 1958: 24. - Bolton, 1986: 255. - Bolton & Stegenga 1987: 168. - Seagrief, 1988, 38: figs. 2,3, 24-26. Joska, 1992: 61-70. - Stegenga, Bolton & Anderson, 1997: 95, Pl. 17. Kandjengo. 2000: 10. Steyn, 2000: 22-31.

***Ulva uncialis* (Kützing) Montagne**

Phycoseris uncialis (Kützing), 1849: 475 (type locality: "ad oras capense" [South Africa]).

Ulva uncialis (Kützing) Montagne, 1850: 246. - Barton, 1893: 54. - Pocock, 1958: 24 ('cf. *uncialis*). Seagrief, 1988: 38, fig. 5:1. - Joska, 1992: 72.

Taxonomic synonym: according to Silva *et al*. (1996)

Ulva capensis Areschoug, 1851: 15-16, *nom illeg*. (syntype localities: Table Bay Algoa Bay, South Africa) (including

Indian Ocean records). Areschoug, 1854: 369-370. - Bolton & Stegenga, 1987: 168.
Manuscript name (*fide* Papenfuss notes):
Ulva minuta Papenfuss *ined.* - Stephenson, 1948: 297. - Isaac, 1953: 63, 71.

Enteromorpha Link, *nom. Cons.*

Enteromorpha compressa (Linnaeus) Nees

Ulva compressa Linnaeus, 1753: 1163 (type locality: Europe).
Enteromorpha compressa (Linnaeus) Nees, 1820: Index [2]. - Krauss, 1846: 215. Kützing, 1849: 480. Barton, 1893: 54. Delf & Michell, 1921: 92. - Seagrief, 1980: 20. Seagrief, 1988: 38, fig. 5:1. - Farrell, Critchley & Aken, 1993: 149. Stegenga, Bolton & Anderson, 1997: 84, 10

Enteromorpha flexuosa (Wulfen in Roth) J. Agardh

Conferva flexuosa Roth 1800: 188 - 190, *nom. Illeg.* (type locality: Diuno, near Trieste, Italy).
Ulva flexuosa Wulfen, 1803: 1
Enteromorpha flexuosa (Wulfen) J. Agardh, 1883: 126-128. - Stegenga, Bolton & Anderson, 1997: 84, 10.

Enteromorpha intestinalis (Linnaeus) Nees

Ulva intestinalis Linnaeus, 1753: 1163 (type locality: " in Mari omni").
Enteromorpha intestinalis (Linnaeus) Nees, 1820: Index [2]. - Barton, 1893: 54. - Delf & Michell, 1921: 92. - Seagrief, 1988: 38, fig. 5:1. - Branch *et al.*, 1994: 308, pl. 146.2. - Stegenga, Bolton & Anderson, 1997: 86, 11.

Enteromorpha linza (Linnaeus) J. Agardh

Ulva linza, Linnaeus, 1753: 1163 (type locality: "in Oceano").
Enteromorpha linza (Linnaeus) J. Agardh, 1883: 134. - Stegenga, Bolton & Anderson, 1997: 86, 12.